Committee for Risk Assessment

RAC

Annex 1

Background document

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

Flumioxazin (ISO); N-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2H- 1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide

EC number: -

CAS number: 41483-43-6

CLH-O-0000004153-83-03/F

Adopted

06 June 2014

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.

CLH report

Proposal for Harmonised Classification and Labelling

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

Substance Name: Flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide

EC Number: -

CAS Number: 103361-09-7

Index Number: 613-166-00-x

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Dossier prepared by Sumitomo Chemical Co., Ltd in accordance with Article 37(6) of CLP.

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Version number: 3	**Date:** 2. January 2013
Version number : 4	**Date :** 26 April 2013
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Part A

TABLES

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

TABLE 1: SUBSTANCE IDENTITY

Substance name:	Flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide
EC number:	-
CAS number:	103361-09-7
Annex VI Index number:	613-166-00-x
Degree of purity:	96.0% (w/w) (equivalent to 960 g/kg)
Impurities:	Confidential information. None of toxicological concern

1.2 Harmonised classification and labelling proposal

Flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide was included in Annex I of Commission Directive 2001/59/EEC adapting to technical progress for the 28th time Council Directive 67/548/EEC, 6th August 2001. The classification "Repr. Cat. 2; R61" was based on developmental effects in the rat and presumed relevance to humans (refer to Part B, Section 4.11 for details). An extensive program of research with flumioxazin has successfully elucidated the mechanism of the developmental toxicity in rats and determined its relevance to humans. The results of this research provide evidence that the rat is particularly sensitive to the toxic effects of flumioxazin whereas this is unlikely to be the case in humans. Therefore, a proposal to change the current harmonised classification and labelling of flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide has been prepared. This proposal focuses on the change in classification of flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide related to reproductive toxicity and therefore, this proposal only includes data relevant to the assessment of this hazard class.

TABLE 2: THE CURRENT ANNEX VI ENTRY AND THE PROPOSED HARMONISED CLASSIFICATION

	CLP Regulation	Directive 67/548/EEC (Dangerous Substances Directive; DSD)
Current entry in Annex VI, CLP Regulation	Repr. 1B, H360D Aquatic Acute 1; H400, Aquatic Chronic 1; H410 M factor acute = 1000,	Repr. Cat. 2, R61 N; R50-53 N; R50-53: C ≥ 0,025 % N; R51-53: 0,0025 % ≤ C < 0,025 % R52-53: 0,00025 % ≤ C < 0,0025 %
Current proposal for consideration by RAC	Removal of Repr. 1B H360D (May damage the unborn child) Addition of M factor chronic = 1000	Removal of Repr. Cat. 2; R61 (May cause damage to the unborn child)
Resulting harmonised classification (future entry in Annex VI, CLP	Aquatic Acute 1; H400, Aquatic Chronic 1; H410	N; R50-53 N; R50-53: C ≥ 0,025 % N; R51-53: 0,0025 % ≤ C < 0,025 %

Regulation)	M factor acute = 1000, M factor chronic = 1000	R52-53: 0,00025 % ≤ C < 0,0025 %

*

1.3 Proposed harmonised classification and labelling based on CLP Regulation and DSD criteria

The proposed classification and labelling of flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide based on the removal of the classification for reproductive toxicity is provided in Table 3 and Table 4.

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)]
3.1.	Acute toxicity - oral	No classification	-	No classification	Conclusive but not sufficient for classification
	Acute toxicity – dermal	No classification	-	No classification	Conclusive but not sufficient for classification
	Acute toxicity - inhalation	No classification	-	No classification	Conclusive but not sufficient for classification
3.2.	Skin corrosion / irritation	No classification	-	No classification	Conclusive but not sufficient for classification
3.3.	Serious eye damage / eye irritation	No classification	-	No classification	Conclusive but not sufficient for classification
3.4.	Respiratory sensitisation	No classification	-	No classification	Conclusive but not sufficient for classification
3.4.	Skin sensitisation	No classification	-	No classification	Conclusive but not sufficient for classification
3.5.	Germ cell mutagenicity	No classification	-	No classification	Conclusive but not sufficient for classification
3.6.	Carcinogenicity	No classification	-	No classification	Conclusive but not sufficient for classification
3.7.	Reproductive toxicity	No classification	-	Repr. 1B (Hazard statement: H360D: May damage the unborn child)	Conclusive but not sufficient for classification
3.8.	Specific target organ toxicity –single exposure	No classification	-	No classification	Conclusive but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure	No classification	-	No classification	Conclusive but not sufficient for classification

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
3.10.	Aspiration hazard	No classification	-	No classification	Conclusive but not sufficient for classification
4.1.	Hazardous to the aquatic environmen		Addition of M (chronic) = 1000	Aquatic acute 1 Aquatic chronic 1 M (acute) = 1000	
5.1.	Hazardous to the ozone layer	No classification	-	No classification	Data lacking

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

Proposed labelling:

Signal word: Warning

Hazard pictogram:

Hazard statements: H410: Very toxic to aquatic life with long lasting effects.

Proposed notes assigned to an entry:

Not applicable

TABLE 4: PROPOSED CLASSIFICATION ACCORDING TO DSD

Hazardous property	Proposed classification	Current classification [1)]	Reason for no classification [2)]
Acute toxicity	No classification	No classification	Conclusive but not sufficient for classification
Acute toxicity – irreversible damage after single exposure	No classification	No classification	Conclusive but not sufficient for classification
Repeated dose toxicity	No classification	No classification	Conclusive but not sufficient for classification
Irritation / Corrosion	No classification	No classification	Conclusive but not sufficient for classification
Sensitisation	No classification	No classification	Conclusive but not sufficient for classification
Carcinogenicity	No classification	No classification	Conclusive but not sufficient for classification
Mutagenicity – Genetic	No classification	No classification	Conclusive but not

Hazardous property	Proposed classification	Current classification [1)]	Reason for no classification [2)]
toxicity			sufficient for classification
Toxicity to reproduction – fertility	No classification	No classification	Conclusive but not sufficient for classification
Toxicity to reproduction – development	No classification	Repr. Cat. 2; R61 May cause harm to the unborn child	Conclusive but not sufficient for classification
Toxicity to reproduction – breastfed babies. Effects on or *via* lactation	No classification	No classification	Conclusive but not sufficient for classification
Environment		N; R50-53	

[1)] Including SCLs
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Proposed labelling:

Indication of danger:

DANGEROUS FOR THE ENVIRONMENT (N)

R-phrases: R50/R53 very toxic to aquatic organisms, may cause long term adverse effects to the aquatic environment

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

Flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2H-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide was included in Annex I of Commission Directive 2001/59/EEC adapting to technical progress for the 28th time Council Directive 67/548/EEC, 6th August 2001.The classification “Category 2; R61 May cause damage to the unborn child” is based on effects observed in the rat developmental toxicity studies.

2.2 Short summary of the scientific justification for the CLH proposal

The previous studies are considered to be adequate in assessing the developmental toxicity of flumioxazin, however since the initial inclusion of flumioxazin further mechanistic work has been undertaken to demonstrate the effects observed in the rat (embryolethality, teratogenicity (mainly ventricular septal defects and wavy ribs) and growth retardation) are species specific and not considered relevant for humans.

Mechanistic research has established that toxic effects observed in the developmental studies and to a lesser extent the repeat dose studies (haematotoxicity) result from inhibition of the enzyme

protoporphyrinogen oxidase (PPO)[1]. The effects reported in the rat developmental study were observed in the absence of maternal toxicity. In the rabbit developmental study, whilst the administered dose was 100-fold greater and maternal toxicity was observed; no embryolethal or teratogenic effects were observed. There is convincing evidence for a single mode of action causing the developmental toxicities in the rat. The sequence of key biological events in the proposed mode of action has been elucidated. Inhibition of PPO interferes with normal haem synthesis, which causes loss of blood cells leading to fetal anaemia, embryolethality and the development of malformations. Rats are particularly sensitive to the effects of PPO inhibition induced by flumioxazin in erythroblasts. This leads to anaemia that is a critical precursor of the developmental toxicity resulting from flumioxazin exposure. The systemic dose-response for this key event has proved to be very steep: half-dose has been without any effect.

In contrast, humans are unlikely to develop anaemia resulting from inhibition of PPO. This conclusion is based on (1) clinical findings that PPO deficient patients with Variegate Porphyria show no signs of anaemia, (2) experimental evidence that flumioxazin does not reduce haem production in K562 cells, which are derived from human erythroleukemia, and (3) that humans are less sensitive to PPO inhibition than rats.

Pharmacokinetic modelling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at exposure equivalent to a maternal dose exceeding 1000 mg/kg/day, thus demonstrating the large species difference in sensitivity. In addition, as a result of the decrease in absorption rate with oral dose, the systemic daily dose cannot exceed value of approximately 100 mg/kg bw.

Overall, it is concluded that the rat is an inappropriate model for assessing the developmental toxicity of flumioxazin in humans because, unlike humans, they are highly sensitive to PPO inhibition, resulting in fetal anaemia and consequent developmental toxicity. There is considered to be no plausible scenario whereby humans would be at risk of developmental toxicity given the species differences in susceptibility to flumioxazin and potential for anaemia.

According to both Boobis *et al* (2008) and Lavelle *et al* (2012) where a mode of action in animals can be demonstrated and judged to be quantitatively irrelevant to humans this should be integrated in the risk assessment improving both the reliability and validity of the results. Consequently, if the mode of action for reproductive toxicity can be demonstrated to be irrelevant to humans, there is no requirement to classify flumioxazin. There is a convincing weight of evidence to conclude that flumioxazin would not present a reproductive hazard to humans and should not be classified for reproductive toxicity based on the criteria for classification in Regulation EC 1272/2008. Therefore, removal of the current reproductive toxicity classification is warranted.

The 2nd ATP to CLP brought in new criteria for classification of long-term hazards to the aquatic environment (e.g. use of chronic toxicity data in classification and separate M factors for acute and chronic toxicity). The environmental hazard assessment was performed in order to determine the chronic M-factor, currently not included in Annex VI of CLP Regulation.

Environment CLH proposal justification:

[1] PPO is responsible for the 7th step in haem production, by removing hydrogen atoms from protoporphyrinogen IX to form protoporphyrin IX. Ultimately protoporphyrin IX forms haem in the 8th step of haem production *via* ferrochelatase.

Aquatic acute category 1 (H400) follows from the acute toxicity of the active substance to *Lemna gibba*: EC_{50} < 1 mg a.s./L (EC_{50} = 0.00035 mg a.s./L, Hoberg, 1996b). A M-factor of 1000 is applicable based on 0.0001 < LC_{50} ≤0.001 mg a.s./l.

Aquatic chronic category 1 (H410) follows from the chronic toxicity of the active substance to *Navicula pelliculosa*: NOEC ≤ 1 mg a.s./L (NOEC < 0.000042 mg/L, Hoberg, 1996a) and the fact that the active substance is not readily biodegradable and not rapidly biodegradable. A M-factor of 1000 is applicable based on 0.00001 < NOEC ≤ 0.0001 mg/l.

R50 follows from the acute toxicity of the active substance to the most sensitive tested aquatic organisms with EC_{50} < 1 mg a.s./L (*Lemna gibba*: EC_{50} = 0.00035 mg a.s./L, Hoberg, 1996b).

R53 follows from the fact that the active substance is not readily biodegradable.

2.3 Current harmonised classification and labelling

2.3.1 CURRENT CLASSIFICATION AND LABELLING IN ANNEX VI, TABLE 3.1 IN THE CLP REGULATION

Classification

Repr. 1B, H360D (May damage the unborn child),

Aquatic Acute 1; H400,

Aquatic Chronic 1; H410,

M factor acute = 1000.

Labelling

Signal word: Danger

Hazard pictogram:
GHS08

GHS09

Hazard statements: H360D: May damage the unborn child

H410: Very toxic to aquatic life with long lasting effects

2.3.2 CURRENT CLASSIFICATION AND LABELLING IN ANNEX VI, TABLE 3.2 IN THE CLP REGULATION

Classification

T; R61 (May cause harm to the unborn child)

N: R50-53

Labelling

Indication of danger:	 Toxic (T)	 Dangerous for the environment (N)
R-phrases:	Repr. Cat. 2; R61 May cause harm to the unborn child N: R50-53	

2.4 Current self-classification and labelling

2.4.1 CURRENT SELF-CLASSIFICATION AND LABELLING BASED ON THE CLP REGULATION CRITERIA

Classification

As *per* the Annex VI entry

2.4.2 CURRENT SELF-CLASSIFICATION AND LABELLING BASED ON DSD CRITERIA

Classification

As *per* the Annex VI entry

Labelling

As *per* the Annex VI entry

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

There are data available to show that the existing harmonised classification for flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide, Repr. 1B (H360D) in accordance with CLP (Repr. Cat. 2; R61 in accordance with Dir 67/548/EEC) is incorrect. Therefore, action is required at the Community level and this proposal seeks to amend the existing entry in Annex VI.

This proposal has been prepared by Sumitomo Chemical Co., Ltd. in accordance with Article 37(6) of CLP and submitted by the Czech Republic.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

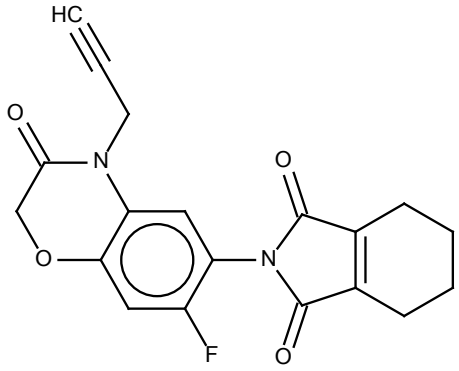
1IDENTITY OF THE SUBSTANCE

1.1 NAME AND OTHER IDENTIFIERS OF THE SUBSTANCE

TABLE 5: SUBSTANCE IDENTITY

EC number:	-
EC name:	Flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide
CAS number (EC inventory):	103361-09-7
CAS number:	103361-09-7
CAS name:	2-[7-fluoro-3,4 –dihydro-3-oxo-4-(2-propynyl)-2*H*-1,4- benzoxazin-6-yl]-4,5,6,7-tetrahydro-1*H*-isoindole-1,3 (2*H*)- dione
IUPAC name:	*N*-(7-fluoro-3,4 –dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4- benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide
CLP Annex VI Index number:	613-166-00-x
Molecular formula:	$C_{19}H_{15}FN_2O_4$
Molecular weight range:	354.33

Structural formula:



1.2COMPOSITION OF THE SUBSTANCE

TABLE 6: CONSTITUENTS (NON-CONFIDENTIAL INFORMATION)

Constituent	Minimum concentration	Concentration range	Remarks
Flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide	96.0% (w/w) (equivalent to 960 g/kg)	96.0 - 100% (w/w)	

Current Annex VI entry: flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide Annex VI index number 613-166-00-x.

Details on the current classification are referred to in Part A, Section 2.3. There are M-factors associated with flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide and there are no notes associated with its Annex VI entry.

TABLE 7: IMPURITIES (NON-CONFIDENTIAL INFORMATION)

Impurity	Minimum concentration	Concentration range	Remarks
-	-	-	No impurities of toxicological concern

Current Annex VI entry: Not applicable

The manufacturer has requested that the impurity profile remains confidential, therefore this information is presented in the IUCLID 5 technical dossier only. The minimum purity of flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide is 96.0% and there are ten process impurities present. These have been taken into consideration in the classification and are not considered to be of additional concern.

TABLE 8:ADDITIVES (NON-CONFIDENTIAL INFORMATION)

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

Current Annex VI entry: Not applicable

1.2.1.Composition of test material

Where available, the purity of the tested material is provided in the relevant sections. The reported studies are considered to be representative of the material as specified above.

1.3 PHYSICO-CHEMICAL PROPERTIES

TABLE 9: SUMMARY OF PHYSICO - CHEMICAL PROPERTIES

Property (guideline[1], GLP status)	Value	Comment[2]	Remarks[3]	Reference
State of the substance at 20°C and 101,3 kPa (Guideline n/a; GLP)	White powdery solid	Visual	1 (reliable without restriction) **key study** **purity: 99.6%**	Foster (2011)
Melting/freezing point (OECD 102, EEC A.1; GLP)	203.51 – 209.74°C	Measured		
Boiling point OECD 103, EEC A.2; GLP)	Could not be determined due decomposition at 273°C	Measured		
Relative density (OECD 109, EEC A.3; GLP)	1.4157 g/cm^2 (20.1°C)	Measured		
Vapour pressure (OECD 104 (gas saturation method), GLP)	0.00032 Pa (22°C)	Measured	1 (reliable without restriction) **key study** **purity: 99.5%**	Pesselman (1990)
Surface tension (EEC A.5; GLP)	70.9 mN/m (20°C)	Measured	1 (reliable without restriction) **key study** **purity: 99.0%**	Wells (1999)
Water solubility (OECD 105, EEC A6 (column elution method); GLP)	0.786 ±0.1081 mg/L (20°C)	Measured	1 (reliable without restriction) **key study** **purity: 99.6%**	Foster & Moseley (2011)
Partition coefficient n-octanol/water (OECD 107 (shake flask method); GLP)	Log P_{ow} = 2.55 (20°C, pH 5.92 – 5.98)	Measured	1 (reliable without restriction) **key study** **purity: 99.9%**	Yamada *et al* (1990)
Flash point (n/a)	n/a	Not measured as flumioxazin is a solid with m.p >40°C	-	-

Property (guideline[1], GLP status)	Value	Comment[2]	Remarks[3]	Reference
Flammability (EEC A.10; GLP)	Classified as not highly flammable, no ignition observed	Measured	1 (reliable without restriction) **key study** **purity: 99.4%**	Russell (1994a)
Explosive properties (EEC A.14, GLP)	Flumioxazin did not explode under the test conditions		1 (reliable without restriction) **key study** **purity: 97.6%**	Sweetapple (1990)
Auto-flammability (EU method A.16; GLP)	No auto-flammability occurred up to 420°C	Measured	1 (reliable without restriction) **key study** **purity: 99.4%**	Russell (1994b)
Oxidising properties (Theoretical assessment; non-GLP)	n/a	Not measured as flumioxazin does not contain functional gps associated with oxidising/reducing activity	-	Radcliffe (1993)
Dissociation constant (OECD 112; Non-GLP)	n/a	Not measured as flumioxazin decomposed at pH>9 and no spectral changes were observed at pH≤7.	1 (reliable without restriction) **key study** **purity: 99.5%**	Furuta (1991)
Viscosity (US EPA OPPTS 830.7100; GLP)	n/a	Not measured n/a as flumioxazin is a solid	-	-

1.Where appropriate, methods employed were guideline compliant
2.Measured or estimated
3.Studies evaluated according the criteria set out by Klimisch *et al* (1997)

2MANUFACTURE AND USES

2.1MANUFACTURE

Flumioxazin is manufactured in Japan.

2.2IDENTIFIED USES

Flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide is a herbicide with a long history of agricultural use for the pre-emergence control of many annual broad-leaved weeds and some annual grasses.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide is not classified with respect to physico-chemical properties. This is not considered further in this dossier.

4 HUMAN HEALTH HAZARD ASSESSMENT

The focus for this classification proposal is the systematic evaluation of the reproductive hazard potential of flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide and consequently the following human health hazard assessment is restricted to information relevant to the proposal, including additional information on repeat dose toxicity, toxicokinetics during gestation, *in vitro* toxicity and reproductive toxicity. All unpublished regulatory guideline studies conducted before and after flumioxazin (ISO); *N*-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2*H*-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide was last reviewed for harmonised classification in 2001 are summarised. Where relevant, published studies from the scientific literature are also summarised and those published after 2001 are highlighted as new information.

The information provided in sections 4.7 and 4.10 are used only as supportive data for toxicity to reproduction.

4.1 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Refer to Section 4.12.1.

4.2 ACUTE TOXICITY

Not relevant for this proposal.

4.3 SPECIFIC TARGET ORGAN TOXICITY – SINGLE EXPOSURE (STOT SE)

Not relevant for this proposal.

4.4 IRRITATION

Not relevant for this proposal.

4.5 CORROSIVITY

Not relevant for this proposal.

4.6 SENSITISATION

Not relevant for this proposal.

4.7 REPEATED DOSE TOXICITY

The results of the subchronic repeat dose toxicity studies considered relevant to this proposal are summarised. For convenience the chronic toxicity results in the combined chronic toxicity and carcinogenicity study in the rat are summarised in Section 4.10.

4.7.1 NON-HUMAN

4.7.1.1REPEATED DOSE TOXICITY: ORAL

The results of experimental studies on repeat dose after oral exposure are summarised in the following table:

TABLE 10: OVERVIEW OF EXPERIMENTAL STUDIES ON REPEATED DOSE TOXICITY AFTER ORAL ADMINISTRATION

Method	Results	Remarks[1]	Reference
90d, rat (SD) (12 animals/sex/gp) oral: feed 0, 30, 300, 1000, 3000 ppm [equiv. 0, 2.3, 20.7, 69.7, 243.5 (M) and 0, 2.2, 21.7, 71.5, 229.6 mg/kg/d (F)] EPA OPP 82-1, GLP	NOAEL: *ca.* 1000 ppm (male/female) based on changes in haematological parameters along with increased incidences of extramedullary haematopoiesis in the spleen and increased absolute spleen weights, relative liver and spleen weights in both males and females at 3000 ppm	1 (reliable without restriction) **key study** **purity: 98.4%**	Hagiwara (1989) SBT-91-0002
90d, rat (SD) (10 animals/sex/gp) oral: feed 0, 30, 300, 1000, 3000 ppm [equiv. 0, 1.9, 19.3, 65.0, 196.7 (M) and 0, 2.2, 22.4, 72.9, 218.4 mg/kg/d (F)] EPA OPP 82-1, GLP	NOAEL: *ca.* 300 ppm (male) based on increased liver, heart, kidney and thyroid weights NOAEL: *ca.* 30 ppm (female) based on haematological changes (including anaemia and extramedullary haematopoiesis	1 (reliable without restriction) **key study** **purity: 94.8%**	Adachi (1991) SBT-10-0023

1.Studies evaluated according the criteria set out by Klimisch *et al* (1997)

90-day oral studies in rats

In first 90-day study (Hagiwara, 1989 SBT-91-0002), five groups, 4 treatment and 1 control group of 12 animals/ sex/group were fed flumioxazin in the diet for 13 consecutive weeks. Dietary concentrations of 0, 30, 300, 1000 and 3000 ppm were administered, equivalent to compound intakes of 0, 2.3, 20.7, 69.7 and 243.5 mg/kg/day for males and 0, 2.2, 21.7, 71.5 and 229.6 mg/kg/day for females, respectively.

Haematology and bone marrow examination results indicated anaemia (decreased haemoglobin concentration (Hb), decreased haematocrit, increased reticulocyte count, increased erythroblast count, decreased red blood cell (RBC) count) in the 3000 ppm group. Increased platelet count and decreased mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV)

were observed in the 1000 and 3000 ppm (both sexes) groups. Increased spleen weights and spleen-to-body weight ratios were observed in the 3000 ppm group (both sexes). Histopathological examination revealed a high incidence of extramedullary haematopoiesis (slight to moderate) of the spleen in the 3000 ppm gp (12/12 M and 8/12 F). Other findings in the study were considered unrelated to treatment.

TABLE 11: SUMMARY OF HAEMATOLOGICAL CHANGES IN THE HAGIWARA (1989) 90-DAY STUDY

Parameter	Male					Female				
Dose level (ppm)	0	30	300	1000	3000	0	30	300	1000	3000
Dose level: approximate equivalent mg/kg/d	0	2.3	20.7	69.7	243.5	0	2.2	21.7	71.5	229.6
Erythrocyte count ($x10^6/\mu L$)	9.34	9.36	9.47	9.50	9.41	8.75	8.30	8.56	9.11	7.77**
Hb conc. (g/dL)	15.78	15.92	15.98	15.71	14.53**	15.26	14.80	15.38	14.76	11.42**
Haematocrit (%)	48.64	48.98	49.37	47.99	44.48**	48.33	45.63	46.94	45.82	36.25**
MCV (fL)	52.11	52.51	52.13	50.53*	47.28**	55.28	55.19	54.90	50.26**	46.79**
MCH (pg)	16.93	17.06	16.97	16.55	15.46**	17.56	17.91	18.00	16.24*	14.75**
MCHC (%)	32.48	32.53	32.55	32.75	32.75	31.77	32.48	32.80	32.35	31.51
Platelet count ($x10^6/\mu L$)	1.30	1.33	1.38	1.45	1.80**	1.26	1.25	1.13	1.55**	2.13**
Erythroblast ratio (/100WBC)	0.4	0.7	0.5	0.5	7.8**	0.4	0.3	0.2	1.1	31.3**
Reticulocyte (%)	0.86	0.81	0.79	1.13	1.68**	1.21	1.47	0.80**	1.11	3.10**

* $p<0.05$, ** $p<0.01$

Based on the results of this study, the NOAEL was 1000 ppm (69.7 mg/kg/day for males and 71.5 mg/kg/day for females) based on changes in haematological parameters along with increased incidences of extramedullary haematopoiesis in the spleen and increased absolute spleen weights, relative liver and spleen weights in both males and females at 3000 ppm.

In a second 90-day study (Adachi, 1991 SBT-10-0023), five groups, 4 treatment and 1 control group of 10 animals/ sex/group, were fed flumioxazin in the diet for 13 consecutive weeks. A further 6 animals/sex/group were fed the diet for 5 consecutive weeks before an interim sacrifice. Dietary concentrations of 0, 30, 300, 1000 and 3000 ppm were administered, equivalent to compound intakes of 0, 1.9, 19.3, 65.0, and 196.7 mg/kg/day for males and 0, 2.2, 22.4, 72.9 and 218.4 mg/kg/day for females, respectively.

Toxic changes observed were most prominently associated with changes in the haematopoietic system. Females were more susceptible to this than males. Haematology and bone marrow examination results indicated microcytic and hypochromic anaemia (decreased Hb concentration, decreased haematocrit, decreased MCHC, increased reticulocyte count, increased erythroblast count and decreased RBC count) at 1000 ppm and greater. Decreased

MCV and MCH were also observed at 300ppm in females. The anaemia was associated with acceleration of the haematopoiesis, such as increased reticulocytes and erythroblasts in the blood, hypercellularity and decreased myeloid/erythroid ratio in the bone marrow. Extramedullary haematopoiesis in the liver and spleen were thought to be related and secondary effects to the anaemia. Pigmentation in the liver might have resulted from increased erythrocyte destruction and increases in heart weight might be regarded as compensatory hypertrophy resulting from lasting anaemia.

TABLE 12: SUMMARY OF HAEMATOLOGICAL CHANGES IN THE ADACHI (1991) 90-DAY STUDY

Parameter	Male					Female				
Dose level (ppm)	0	30	300	1000	3000	0	30	300	1000	3000
Dose level: approximate equivalent mg/kg/d	0	1.9	19.3	65.0	196.7	0	2.2	22.4	72.9	218.4
INTERIM SACRIFICE (WEEK 5)										
Erythrocyte count ($x10^6/\mu L$)	7.78	7.80	7.91	8.04	8.20	7.66	7.79	7.63	8.04	7.23*
Hb conc. (g/dL)	14.9	14.7	14.8	13.9**	13.3**	14.6	14.6	14.0	13.1**	10.6**
Haematocrit (%)	40.8	40.7	41.1	39.1	38.7*	38.8	39.2	37.6	35.9**	30.3**
MCV (fL)	52.5	52.2	52.0	48.7**	47.2**	50.7	50.3	49.4	44.6**	41.9**
MCH (pg)	19.1	18.8	18.7	17.3**	16.3**	19.1	18.8	18.4	16.2**	14.7**
MCHC (g/dL)	36.3	36.0	35.9	35.5**	34.5**	37.7	37.4	37.3	36.3**	35.0**
Neutrophil count ($10^3/\mu L$)	0.76	0.60	0.97	0.63	0.77	0.50	0.46	0.47	0.48	0.96**
Erythroblast ratio (/100WBC)	0	0	0	1	4**	1	0	0	8	53**
Reticulocyte (‰)	16.0	18.8	17.4	21.9	35.9**	7.6	10.0	9.8	22.9	73.1**
WEEK 13										
Erythrocyte count ($x10^6/\mu L$)	8.34	8.39	8.59	8.61	9.00**	7.65	7.94	7.98	8.38	6.51
Hb conc. (g/dL)	14.6	14.8	14.8	13.9**	13.6**	13.7	14.1	13.8	12.4	8.8**
Haematocrit (%)	39.7	39.8	40.6	38.4	37.9*	38.0	38.9	38.2	35.9	26.6**
MCV (fL)	47.6	47.4	47.3	44.6**	42.2**	49.6	49.0	47.9*	42.9**	40.7**
MCH (pg)	17.5	17.6	17.3	16.2**	15.1**	17.9	17.7	17.3*	14.9**	13.4**
MCHC (g/dL)	36.8	37.2	38.5	36.3*	35.9**	36.2	36.1	36.0	34.6**	32.8**

Parameter	Male					Female				
Dose level (ppm)	0	30	300	1000	3000	0	30	300	1000	3000
Dose level: approximate equivalent mg/kg/d	0	1.9	19.3	65.0	196.7	0	2.2	22.4	72.9	218.4
Neutrophil count (10^3/ µL)	0.68	0.89	1.88	0.72	0.77	0.48	0.46	0.45	0.42	0.92*
Erythroblast ratio (/100WBC)	0	0	1	1	5**	0	0	0	9	103**
Reticulocyte (‰)	7.8	9.0	6.7	9.7	13.8**	9.7	8.1	9.7	17.4*	38.9**

* $p<0.05$, ** $p<0.01$

Based on the results of this study, the NOAEL was considered to be 30 ppm (2.2 mg/kg/day) based on haematological changes (including anaemia and extramedullary haematopoiesis) in females. For males, the NOAEL was considered to be 300 ppm (19.3 mg/kg/day) based on increased liver, heart, kidney, thyroid weights and evidence of low grade anaemia.

4.7.1.2REPEATED DOSE TOXICITY: INHALATION

Not relevant for this proposal.

4.7.1.3REPEATED DOSE TOXICITY: DERMAL

The results of experimental studies on repeat dose after dermal exposure are summarised in the following table:

TABLE 13: OVERVIEW OF EXPERIMENTAL STUDIES ON REPEATED DOSE TOXICITY AFTER DERMAL EXPOSURE

Method	Results	Remarks[1]	Reference
21d, rat (SD) (5 animals/sex/gp) dermal: semi-occuled 0, 100, 300, 1000 mg/kg/day EPA OPP 82-2, GLP	NOAEL: > 1000 mg/kg/day (male) based on no adverse effects observed up to the maximum dose tested NOAEL: ca. 300 mg/kg/day (female) based on decreased mean Hb and haematocrit values	1 (reliable without restriction) **key study** **purity: 94.8%**	Osheroff (1991) SBT-11-0026

1.Studies evaluated according the criteria set out by Klimisch *et al* (1997)

21-day dermal toxicity study (SBT-11-0026)

Four groups, 3 treatment and 1 control group of 5 animals/ sex/group, had flumioxazin administered dermally in corn oil to pre-clipped test sites once daily for 21 days. After application gauze was applied and wrapped to prevent loss of dose and potential oral ingestion. Following a 6 h exposure dressings were removed and the test site wiped using gauze moistened with distilled water. Doses used were 0, 100, 300 and 1000 mg/kg/d.

There were no indications of treatment related effects observed on body weight, food consumption, gross pathology, dermal response, clinical signs of toxicity or biochemical parameters. Signs of toxicity were limited to females in the high dose group with significant decreases in both Hb concentration and haematocrit values.

TABLE 14: SUMMARY OF HAEMATOLOGICAL CHANGES IN THE 21-DAY DERMAL STUDY

Parameter	Male				Female			
Dose (mg/kg bw)	0	100	300	1000	0	30	300	1000
Hb conc. (g/dL)	16.4	16.4	16.6	15.9	16.9	16.2	16.5	15.7*
Haematocrit (%)	48.3	48.5	48.7	46.9	49.1	46.9	48.5	46.1*

* $p<0.05$

Based on the results of this study, the NOAEL was considered to be 1000 mg/kg/d for males (the maximum dose tested). For females the NOAEL was considered to be 300 mg/kg/d based on decreased mean Hb and haematocrit values.

4.7.1.4REPEATED DOSE TOXICITY: OTHER ROUTES

No relevant information.

4.7.1.5HUMAN INFORMATION

No relevant information.

4.7.1.6 OTHER RELEVANT INFORMATION

None.

4.7.1.7 SUMMARY AND DISCUSSION OF REPEATED DOSE TOXICITY

Overall, irrespective of the route of administration (oral (dietary) or dermal) toxic changes observed following flumioxazin exposure in the rat are associated with changes in the haematopoietic system. The changes are characteristic of anaemia and generally involve decreased Hb concentration, decreased haematocrit, decrease RBC count along with increases in reticulocytes and erythroblasts in the blood. Due to the decrease in the parameters mentioned, the compensatory mechanism involves hypercellularity and decreased myeloid : erythroid ratio in the bone marrow accompanied with acceleration of haematopoiesis (hence the increase in circulating immature RBC (i.e. reticulocytes and erythroblasts). Secondary effects to the anaemia involve extramedullary haematopoiesis in the liver and spleen, with evidence of increased erythrocyte destruction in the liver (manifest as pigmentation of the liver) and potential compensatory hypertrophy of the heart.

Repeat dose toxicity studies showed that the rat was the most susceptible species and there was no evidence of anaemia in either the mouse (28-day) or dog (90-day and 1 year) studies (these data have not been reported in this proposal as they did not provide any evidence of toxicity relevant for the mode of action proposed in the rat). However, the absence of haematological effects in these other species is discussed later in the context of species differences in the toxicity of flumioxazin.

Further discussion of the mode of action of the anaemia is presented in Section 4.12.2.

4.8 SPECIFIC TARGET ORGAN TOXICITY (CLP REGULATION) – REPEATED EXPOSURE (STOT RE)

Not relevant for this proposal.

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

Summary of the Dossier submitter's proposal

The DS did not make a proposal for specific target organ toxicity (repeated exposure) and information on repeated dose toxicity was only included in the CLH report due to its relevance to the reproductive toxicity classification proposal. However, the haematotoxicity of flumioxazin was raised by one member state (MS) during public consultation and a discussion on classification for this hazard class is included in this opinion for this reason. Following this comment, RAC accessed this hazard class.

Two 90-day dietary studies, a 21-day dermal study and a 52/104 week feeding study, all conducted in the SD rat were summarised. Although sub-chronic data were also available for the mouse and dog, these were considered not relevant with respect to reproductive toxicity in the rat and were therefore not presented.

90-day dietary studies:

Dietary levels of 0, 30, 300, 1000 and 3000 ppm were used in both 90 day studies. In the first study (Hagiwara, 1989), doses used were equivalent to 0, 2.3, 20.7, 69.7 and 243.5 mg/kg/day (males) and 0, 2.2, 21.7, 71.5 and 229.6 mg/kg/day (females). Significant adverse effects on the blood were seen at the high dose. Anaemia was indicated by a decreased haemoglobin concentration (Hb) (7.9% in males, 25% in females), decreased haematocrit, increased reticulocyte count, increased erythroblast count and decreased red blood cell (RBC) count. In addition, a high incidence of extramedullary haematopoiesis (slight to moderate) of the spleen occurred in the high dose males and females and there was an increase in absolute spleen weights as well as relative liver and spleen weights in both males and females. Increased platelet count ($p<0.01$ in males) and decreased mean corpuscular haemoglobin (MCH) (non-significant) and mean corpuscular volume (MCV) ($p<0.05$) were seen at the 1000 ppm (69.7/71.5 mg/kg) dose.

In the second study (Adachi, 1991), significant toxicity to the blood was seen from ≥ 1000 ppm (65/72.9 mg/kg). Microcytic and hypochromic anaemia was indicated by decreased Hb concentration (by 6.8% in males and 37.6% in females at 3000 ppm and by 4.8% in males and 9.5% in females at 1000 ppm), decreased haematocrit, decreased MCHC, increased reticulocyte count, increased erythroblast count and decreased RBC count. The anaemia was associated with evidence for acceleration of haematopoiesis (in liver from 1000 ppm; in spleen; 1, 8, 10 females at 300, 1000, 3000 ppm), such as increased reticulocytes and erythroblasts in the blood, hypercellularity and decreased myeloid/erythroid ratio in the bone marrow (females ≥ 1000ppm). Extramedullary haematopoiesis in the liver and spleen were likely to be related and secondary effects to the anaemia. Pigmentation in the liver was likely to have resulted from increased erythrocyte destruction and increases in heart weight might be regarded as compensatory hypertrophy resulting from lasting anaemia.

21-day dermal:

The findings of the 90-day studies were supported by the observation of significantly decreased haemoglobin concentration (by 7%) and haematocrit in females at 1000 mg/kg bw/day in the dermal study. Females were clearly more susceptible in these studies.

105 week dietary study:

Doses of 0 to 1000 ppm, equivalent to 0, 1.8, 18.0, and 36.5 mg/kg/day for males and 0, 2.2, 21.8 and 43.6 mg/kg/day for females, respectively, were administered to SD rats in a 2-year study. Haematological changes associated with anaemia were evident in rats of the 500 and 1000 ppm groups, with significant alterations occurring in the high dose females. A reduction in haemoglobin of approximately 10% was recorded at each time point in high dose females. A slight increase ($p<0.05$) in extra medullary haematopoiesis was observed. The anaemia

lasted throughout the treatment period.

Comments received during public consultation

The DS did not make a proposal for repeated dose toxicity and information on this endpoint was included in the CLH report due to its relevance to the reproductive toxicity classification proposal. However, the haematotoxicity of flumioxazin was raised by one MS during public consultation proposing that the repeated toxicity data should be reviewed under the CLP criteria; an assessment by RAC is included here for this reason.

Assessment and comparison with the classification criteria

The key adverse effects relevant for classification for repeated dose toxicity are the haemolytic anaemia seen particularly in female rats following 90-day and 105 week dietary exposure. Some additional details with respect to histopathology were taken from the DAR/Study reports.

	Rat oral data	
Study/dose levels (mg/kg bw/d)	**STOT RE 2**	**Effects at doses ≤ cut-off values**
90-day (1) (Hagiwara, 1989) (0, 30, 300, 1000, 3000 ppm) 0, 2.3, 20.7, 69.7, 243.5 mg/kg/day (males) 0, 2.2, 21.7, 71.5, 229.6 mg/kg/day (females)	*Guidance value: 100 mg/kg bw/d* **= No classification**	*69/71 mg/kg bw/d:* ↓MCH (females) ↓ MCV (♁**/♂*) ↑platelet count (♁**)
90-day (2) (Adachi, 1991) (0, 30, 300, 1000, 3000 ppm) 0, 1.9, 19.3, 65.0, 196.7 mg/kg/day (males) 0, 2.2, 22.4, 72.9, 218.4 mg/kg/day (females)	*Guidance value: 100 mg/kg bw/d* **Classification?**	*65/72.9 mg/kg bw/d*: ↓Hb (4.8%♂/♁9.5%) ↓Haematocrit (**♁) ↓MCHC (**♂/**♁) ↑reticulocytes(↑ns) ↑erythroblasts (↑♁ ns) ↓RBC ↑haematopoiesis ↑liver pigmentation
105 week study (Seki, 1993) (0, 50, 500, 1000 ppm) 0, 1.8, 18.0, 36.5 mg/kg/day (males) 0, 2.2, 21.8 43.6 mg/kg/day (females)	*Guidance value: 12.5 mg/kg bw/d* **Classification?**	*18/21.8 mg/kg bw/d:* ↓Hb (♁9.5%) ↓Haematocrit (**♁) ↓ MCV** ↓MCHC (**♁) ↑reticulocytes(*at wk 14/↑ns other time points) Slight ↑extramedullary haematopoiesis

*P<0.05, **p<0.01, ns: not statistically significant

The table above compares the cut-off values for STOT RE Cat 2 with the findings from relevant sub-chronic studies at or close to these cut-off dose levels.
Severe anaemia was induced at the high dose level in the 90-day rat study (Hagiwara, 1989) at a dose in excess of the cut-off values. The intermediate dose (69/71 mg/kg bw/d) was lower than 100 mg/kg bw/d (STOT RE Cat 2). However, the findings at this dose level were not considered sufficient or severe enough to warrant classification according to the criteria. A

greater degree of haematotoxicity was seen at the same/similar dose level in females of the second 90-day study (Adachi, 1991) where severe anaemia was seen at the high dose and some evidence also at the intermediate dose with females more sensitive; a 9.6% reduction in haemoglobin was accompanied by significant alterations to other red blood parameters and clear evidence of both spleen and liver extramedullary haematopoiesis.

Brown pigmentation (presumably haemosiderin) in hepatocytes, liver canaliculi, and tubular epithelial cells, in addition to hepatocytic degeneration, necrosis and renal tubular cell vacuolation were seen in 3/10 females at the high dose. At 1000 ppm, pigmentation of the sinusoidal cells was seen in only one female, while increased extramedullary haematopoiesis was seen in 8/10 females and hypercellularity of the bone marrow in 6/10 females, while there was no evidence of degenerative change in the liver, kidney or spleen. There was no evidence of haemoglobinurea or haemosiderinurea reported.

In the 105 week rat study, there was evidence of significant anaemia at approximately 20 mg/kg bw/d. However, the guidance value for a long-term study was ≤12.5 mg/kg bw, therefore classification was not supported.

According to the criteria in the CLP Regulation (Annex I, 3.9.1.4), the "assessment shall take into consideration not only significant changes in a single organ or biological system but also generalised changes of a less severe nature involving several organs."
The CLP Guidance provides the following example:
"· **Marked** increase of haemosiderosis in the spleen, liver or kidney in combination with other changes indicating significant haemolytic anaemia (e.g. a reduction in Hb at ≥10%) in a 28 day study.
· **Significant increase** in haemosiderosis in the spleen, liver or kidney in combination with microscopic effects like necrosis, fibrosis or cirrhosis.

The results from the first 90-day study do not support classification according to the CLP criteria.
More marked haematotoxicity was seen at the 69/71 mg/kg bw/d dose in the 2nd 90-day study where the effects were borderline for classification.
-Approximately 10% reduction in Hb,
-some evidence of degeneration in liver and kidney,
-Evidence of haemosiderin in liver and kidney tubular epithelium.

The effects seen in the 105 week study occurred at a dose significantly greater than the guidance value. Even when the relative sensitivity of the rat to haematotoxicity is also considered, the weight of evidence supports no classification. The RAC concludes that no classification is required for STOT RE.

4.9 MUTAGENICITY (GENOTOXICITY)

Not relevant for this proposal.

4.10 CARCINOGENICITY

4.10.1 NON-HUMAN INFORMATION

4.10.1.1CARCINOGENICITY: ORAL

Not relevant for this proposal. The results of the chronic toxicity component of the combined chronic toxicity and carcinogenicity study in the rat are relevant and are summarised in the following table:

TABLE 15: OVERVIEW OF EXPERIMENTAL STUDIES ON CHRONIC TOXICITY AFTER ORAL ADMINISTRATION

Method	Results	Remarks[1]	Reference
52 or 104 wk, rat (SD) (50 animals/sex/gp) oral: feed 0, 50, 500, 1000 ppm [equiv. 0, 1.8, 18.0, 36.5 (M) and 0, 2.2, 21.8, 43.6 mg/kg/d (F)] EPA OPP 83-5, GLP	Non-neoplastic effects: NOAEL: ca. 50 ppm (male/female) (based on: chronic nephropathy (male) and haematological changes (anaemia, both genders)	1 (reliable without restriction) **key study** **purity: 94.8%**	Seki (1993) SBT-30-0040

1.Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Summary of oral chronic toxicity data (SBT-30-0040)

Four groups, 3 treatment and 1 control group of 50 animals/ sex/group were fed flumioxazin in the diet for 24 consecutive months. A further 24 animals/sex/group were fed the diet for 12-18 consecutive months as satellite groups. Dietary concentrations of 0, 50, 500 and 1000 ppm were administered, equivalent to compound intakes of 0, 1.8, 18.0, and 36.5 mg/kg/day for males and 0, 2.2, 21.8 and 43.6 mg/kg/day for females, respectively.

Toxic changes observed were most prominently associated with changes in the haematopoietic system. Haematological changes associated with anaemia in rats of the 500 and 1000 ppm groups. A slight increase ($p<0.05$) in extramedullary haematopoiesis was observed. The anaemia lasted throughout the treatment period; however it was not aplastic in nature. Chronic nephropathy was observed in males in the intermediate and high dose groups.

TABLE 16: SUMMARY OF HAEMATOLOGICAL CHANGES IN THE RAT CHRONIC STUDY

Parameter		Male				Female			
Dose level (ppm)	Wk	0	50	500	1000	0	50	500	1000
Dose level: approximate equivalent mg/kg/d		0	1.8	18.0	36.5	0	2.2	21.8	43.6
Erythrocyte count ($x10^6/\mu L$)	14	8.82	9.06	8.85	9.11	7.99	7.96	8.21	8.46**
	27	8.96	9.00	9.17	9.21	8.07	7.62*	8.28	8.51*
	53	8.65	8.94	8.76	9.10	7.19	7.08	7.51	7.71**
	79	7.94	8.35	8.38	8.43	7.37	6.99	7.13	7.35
	105	7.68	7.41	7.12	7.92	6.37	6.48	7.15	6.54
Hb conc. (g/dL)	14	15.4	15.5	15.0*	14.9*	15.0	14.9	14.0**	13.4**
	27	15.3	15.1	15.4	15.0	15.0	14.7	14.6	13.8**

Parameter		Male				Female			
Dose level (ppm)	Wk	0	50	500	1000	0	50	500	1000
	53	14.2	14.2	13.8	14.1	13.2	12.9	12.7	12.0**
	79	13.6	14.2	13.9	13.7	13.8	13.4	12.6*	12.1**
	105	14.0	13.4	12.5	13.6	12.3	12.3	12.9	11.1
Haematocrit (%)	14	44.1	44.8	43.4	43.1	41.5	41.3	39.6**	38.7**
	27	45.6	45.4	45.8	45.1	44.0	42.8	43.0	41.2**
	53	42.8	43.5	42.5	43.0	38.6	38.0	37.7	36.3
	79	39.1	40.8	40.3	40.3	38.7	37.7	36.7	35.3**
	105	39.3	37.7	35.9	38.5	35.1	34.6	36.7	32.1
MCV (fL)	14	50.0	49.5	49.1	47.4**	52.0	51.9	48.2**	45.8**
	27	50.9	50.4	50.0	49.2	54.5	56.1	52.1**	48.5**
	53	49.5	48.7	48.6	47.3	53.7	53.7	50.2**	47.2**
	79	49.3	48.9	48.1	47.9	52.6	54.1	52.2	48.3*
	105	52.3	50.8	50.3	48.5	55.8	53.9	51.9*	49.7**
MCH (pg)	14	17.4	17.1	17.0	16.4**	18.8	18.7	17.0**	15.9**
	27	17.1	16.8	16.8	16.3**	18.6	19.2*	17.6**	16.1**
	53	16.4	15.9	15.8	15.5**	18.4	18.2	16.9**	15.6**
	79	17.1	17.0	16.6	16.3	18.7	19.2	17.7*	16.6**
	105	18.6	17.8	17.4	17.2	19.4	19.0	18.2*	17.2**
MCHC (g/dL)	14	34.8	34.6	34.5	34.6	36.1	36.1	35.3*	34.7**
	27	33.6	33.4	33.5	33.2	34.2	34.3	33.8	33.4**
	53	33.1	32.7	32.5	32.8	34.2	33.9	33.8	33.1**
	79	34.8	34.8	34.5	34.0**	35.6	35.5	34.2**	34.3*
	105	35.6	35.1	34.6	35.4	34.9	35.3	35.1	34.6
Erythroblast ratio (/100WBC)	14	0	1	0	1	0	1	0	14
	27	0	0	0	1	0	0	2	4**
	53	0	1	0	1	0	1	1	19*
	79	0	1	0	2**	0	0	5	18**
	105	1	0	0	6	1	1	1	25**
Reticulocyte (‰)	14	8.0	10.4	11.0	10.9	11.4	14.6	18.4*	18.7**
	27	18.9	13.1	16.6	16.3	13.7	17.9	19.2	23.1
	53	13.3	14.7	14.0	12.6	12.7	14.3	15.3	17.4
	79	18.2	13.7	15.2	15.2	15.7	16.7	30.9	26.2
	105	23.1	31.4	35.1	21.4	37.9	33.5	24.1	42.5

* $p<0.05$, ** $p<0.01$

Based on the results of this study, flumioxazin was concluded not to be carcinogenic. The NOAEL for chronic toxicity was considered to be 50 ppm (equivalent to 1.8 and 2.2 mg/kg/d,

for males and females respectively) based on chronic nephropathy (males) and haematological changes (anaemia, both genders).

Note: there was no evidence of adverse haematological effects in the mouse carcinogenicity study (SBT-30-0040). Therefore the study is not relevant to this proposal and it has not been summarised.

4.10.1.2CARCINOGENICITY: INHALATION

Not relevant for this proposal.

4.10.1.3CARCINOGENICITY: DERMAL

Not relevant for this proposal.

4.10.1.4HUMAN INFORMATION

None.

4.10.2 OTHER RELEVANT INFORMATION

None.

4.10.3 OVERALL SUMMARY AND DISCUSSION OF CHRONIC TOXICITY DATA

Overall, chronic flumioxazin exposure *via* dietary administration in the rat was associated with increased incidences of extramedullary haematopoiesis and associated anaemia throughout the treatment period. The anaemia however was not aplastic in nature. No haematopoietic changes were observed in the mouse, suggesting that the rat was more susceptible. There were no treatment related oncogenic effects in either species.

Further discussion of the mode of action of the anaemia is presented in Section 4.12.2.

4.11 TOXICITY FOR REPRODUCTION

4.11.1 EFFECTS ON FERTILITY

The results of experimental fertility studies are summarised in the following table:

TABLE 17: OVERVIEW OF EXPERIMENTAL STUDIES ON FERTILITY TOXICITY

Method	Results	Remarks[1]	Reference
rat (SD) two-generation study (30 animals/sex/gp) oral: feed 0, 50, 100, 200, 300 ppm [equiv. P_1: 0, 3.2, 6.3, 12.7, 18.9 mg/kg/d (M) and 0, 3.8, 7.6, 15.1, 22.7 mg/kg/d (F); F_1: 0, 3.7, 7.5, 15.0, 22.4 mg/kg/d (M) and 0, 4.3, 8.5, 17.2, 25.6 mg/kg/d (F)] EPA OPP 83-4 , GLP	NOAEL parental toxicity: *ca*. 200 ppm (male/female) (based on adverse clinical signs, reductions in body weight, body weight gain, food consumption and organ weights) NOAEL offspring toxicity: *ca.* 100 ppm (male/female) (based on reduced pup body weights, increase in stillbirths with viability index and litter size reduced) NOAEL reproductive: *ca*. 200 ppm (female) (based on reduced gestation index in both P_1 and F_1 generations and an increase in the number of F_1 dams that did not deliver a litter)	1 (reliable without restriction) **key study** **purity: 94.8%**	Hoberman, A.M. (1992) SBT-21-0035

1.Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Summary of fertility data (SBT-21-0035)

Flumioxazin was administered *via* the diet to 30 Sprague-Dawley rats/sex/group at dietary concentrations of 0, 50, 100, 200 or 300 ppm in the diet. These doses were equivalent to 0, 3.2, 6.3, 12.7 or 18.9 mg/kg/day for P_1 males and 0, 3.8, 7.6, 15.1 or 22.7 mg/kg/day for P_1 females, respectively. For the F_1 generation, equivalent doses were 0, 3.7, 7.5, 15.0 or 22.4 mg/kg/day for males and 0, 4.3, 8.5, 17.2 or 25.6 mg/kg/day for females. The P_1 and F_1 parents were given the test diets for 12 weeks prior to mating to produce the F_1 and F_2 litters. F_1 pups were weaned at 21 days of age and approximately 30 pups/sex/group were randomly selected as parents of the F_2 generation. Sperm parameters were not evaluated in the parental males and sexual maturation and developmental landmarks were not evaluated in the offspring.

Parental toxicity was evidenced with decreased body weights in F_1 males accompanied by increased food consumption.

TABLE 18: EFFECTS ON PARENTAL ANIMALS (P1 GENERATION)

Parameter	Male					Female				
Dose level (ppm)	0	50	100	200	300	0	50	100	200	300
Dose level: approximate equivalent mg/kg/d	0	3.2	6.3	12.7	18.9	0	3.8	7.6	15.1	22.7
Clinical signs										
Vagina: red substance present						1/28	0/28	1/27	1/30	9/25**
Body weights (g)										
premating Day 83	604.2	619.9	596.2	595.7	613.6	332.3	337.3	328.7	332.9	331.6
gestation Day 20						464.3	471.1	462.6	460.8	416.3**
lactation Day 21						362.3	364.7	369.1	360.9	384.4
terminal	700.1	728.6	698.0	695.8	708.2	367.3	370.5	369.7	372.2	369.7
Food consumption (g/d)										
premating (Day 1-83)	28.8	29.5	28.5	28.5	28.8	20.4	20.9	20.3	20.4	20.4
gestation (Day 0-20)						25.3	25.7	25.8	25.0	25.7
lactation (Day 1-14)						45.3	43.8	45.2	43.9	36.9**

**: Statistically significance ($p<0.01$)

The changes of clinical sign and body weigh gain in the females of the 300ppm group were considered to be attributable to the foetal toxicity.

TABLE 19: EFFECTS ON PARENTAL ANIMALS (F1 GENERATION)

Parameter	Male					Female				
Dose level (ppm)	0	50	100	200	300	0	50	100	200	300
Dose level: approximate equivalent mg/kg/d	0	3.7	7.5	15.0	22.4	0	4.3	8.5	17.2	25.6
Clinical signs										
Total mortality	0/30	1/30	2/30	0/30	1/30	0/30	1/30	0/30	0/30	5/30**
Pale	0/30	0/30	0/30	0/30	3/30**	0/30	0/30	0/30	0/30	3/30**
Vagina: red substance present						0/27	0/28	0/25	0/25	1/20
Body weights (g)										
premating Day 99	616.1	641.4	637.0	612.7	570.1*	347.9	356.8	350.4	345.2	330.0
gestation Day 20						483.0	474.9	479.4	461.8	430.8**
lactation Day 21						385.6	390.0	393.6	387.0	379.1
terminal	692.5	726.4	726.8	688.7	647.6*	388.4	396.2	396.3	383.8	377.0
Food consumption (g/d)										
premating (Day 1-99)	29.5	30.7	31.0*	29.9	27.7*	21.7	22.3	21.5	21.3	20.1**
gestation (Day 0-20)						27.0	26.5	25.8	25.7	25.0
lactation (Day 1-14)						47.6	44.4	46.1	44.2	37.6**
Organ wt (g)										
Epididymis(L)	0.83	0.79	0.85	0.77	0.74**					
(R)	0.83	0.81	0.85	0.77	0.72**					
Testis(L)	1.99	2.01	1.96	1.87	1.82**					
(R)	1.98	2.02	1.96	1.86	1.83**					
Prostate	1.22	1.16	1.16	1.12	1.08					
Necropsy										
Liver: yellow						0/30	0/30	0/30	0/30	3/30**
Histopathology										
Liver:										
bile stasis										2/4
necrosis, centrilobular										3/4

*: Statistically significance ($p<0.05$)
**: Statistically significance ($p<0.01$)

Litters in the 200 ppm group had significant increases in stillbirths with related significant reductions in live born pups in F2 generation. Stillborns were not increased in the high dose groups in F2 generation as embryo-fetal death occurred earlier in gestation. Pup weight was significantly decreased in the pups of 200ppm group in F1 generation. The gestation index (live litters delivered/pregnant females), number of pups delivered, number of liveborn pups, viability index, pup weight and litter size were significantly reduced in the 300ppm group in both generations. The gestation index (and the number of F_1 dams that did not deliver a litter) is related to the embryo-foetal death.

A general reduction in mating performance across all groups including controls was attributed to a genetically mediated problem in the rats supplied by Charles River breeding laboratory. No treatment related effects on fertility were observed.

TABLE 20: LITTER EFFECTS IN THE FERTILITY STUDY (P1 FEMALES – F1 PUPS)

Parameter	Dose level (ppm)				
	0	50	100	200	300
Dose level: approximate equivalent mg/kg/d	0	3.8	7.6	15.1	22.7
Gestation index	23/23(100.0%)	22/22(100.0%)	25/26(96.2%)	28/28(100.0%)	16/21(76.2%)**
Dams with stillborn pups	3/23(13.0%)	3/22(13.6%)	3/26(11.5)	2/28(7.1%)	1/16(4.8%)
Implantations (mean)	16.1 ± 2.8	15.9 ± 3.9	15.5 ± 4.0	16.5 ± 2.6	15.0 ± 3.7
Pups delivered (mean)	14.4 ± 3.6	15.2 ± 4.0	13.8 ± 4.7	14.6 ± 3.8	7.2 ± 5.3**
Live born (mean)	14.2 ± 3.7	14.8 ± 4.0	14.2 ± 4.0	14.5 ± 3.8	7.0 ± 5.1**
Still born (mean)	0.1 ± 0.3	0.3 ± 0.8	0.2 ± 0.7	0.1 ± 0.3	0.2 ± 0.8
Viability index (%)	99.4	98.5	96.3* [a]	95.1** [a]	87.5**
Pup weight (g) D1, post partum	6.9 ± 0.6	6.5 ± 0.5	6.7 ± 0.8	6.1 ± 0.7**	5.7 ± 0.6**

These changes were unrelated to treatment because the values were within the historical control range (93.8-100%)

TABLE 21: LITTER EFFECTS IN THE FERTILITY STUDY (F1 FEMALES – F2 PUPS)

Parameter	Dose level (ppm)				
	0	50	100	200	300
Dose level: approximate equivalent mg/kg/d	0	4.3	8.5	17.2	25.6
Gestation index	23/23(100.0%)	20/21(95.2%)	19/19(100.0%)	18/18(100.0%)	16/18(88.9%)
Dams with stillborn pups	2/23(8.7%)	3/20(15.0%)	2/19(10.5%)	4/18(22.2%)	2/16(12.5%)
Implantations (mean)	17.6 ± 2.9	15.4 ± 5.1	17.3 ± 2.0	15.4 ± 4.7	16.2 ± 3.1
Pups delivered (mean)	15.8 ± 3.6	13.6 ± 5.4	15.7 ± 2.4	13.4 ± 4.4	9.6 ± 5.1**
Live born (mean)	15.6 ± 3.7	13.3 ± 5.3	15.6 ± 2.5	12.8 ± 4.3**	9.4 ± 5.0**
Still born (mean)	0.1 ± 0.3	0.2 ± 0.7	0.1 ± 0.3	0.6 ± 1.3**	0.1 ± 0.3
Viability index (%)	98.6	97.6	97.3	96.5	79.2**
Pup weight (g) D1, post partum	6.4 ± 0.5	6.7 ± 0.6	6.3 ± 0.5	6.1 ± 0.7	5.6 ± 0.4**

Based on the results of this study the NOAEL for parental toxicity was 200 ppm (12.7 mg/kg/d), based on clinical signs of toxicity and reductions in body weight, body weight gain, food consumption and organ weights. The reproductive NOAEL was also considered to be 200 ppm, based on reduced gestation index in both the P_1 and F_1 generations and an increase in the number of F_1 dams that did not deliver a litter in the F_1 generation. The NOAEL for offspring toxicity was 100 ppm (7.6 mg/kg/d) based on reduced pup bodyweights, increase in stillbirths with viability index and litter size reduced and clinical and necropsy effects related to increased pup mortality.

4.11.2 EFFECTS ON DEVELOPMENTAL TOXICITY

4.11.2.1DEVELOPMENTAL TOXICITY: ORAL

The results of rat and rabbit developmental studies are summarised in the following table:

TABLE 22: OVERVIEW OF EXPERIMENTAL STUDIES ON DEVELOPMENTAL TOXICITY AFTER ORAL ADMINISTRATION

Method	Results	Remarks[1]	Reference
rat (SD) developmental study (22 females/gp) oral: gavage 0, 1, 3, 10, 30 mg/kg/d (exposed from GD 6 – 15) EPA OPP 83-3; GLP	NOAEL maternal toxicity: >30 mg/kg/day based on no signs of maternal toxicity observed up to the highest dose tested NOAEL developmental toxicity: *ca*. 10 mg/kg/day based on increased incidence of cardiac ventricular septal defects, wavy ribs, curvature of the scapular and reduced ossification of sacrococcygeal vertebral bodies	1 (reliable without restriction) **key study** **purity: 94.8%**	Kawamura, S. (1990a) SBT-00-0012
rabbit (NZW) developmental study (20 females/gp) oral: gavage 0, 300, 1000, 3000 mg/kg/d (exposed GD 7 – 19) EPA OPP 83-3; GLP	NOAEL maternal toxicity: *ca*. 1000 mg/kg/day based on reductions in maternal body weight gains and relative and absolute food consumption NOAEL developmental toxicity: >3000 mg/kg/day no effects observed up to the highest dose tested	1 (reliable without restriction) **key study** **purity: 94.8%**	Hoberman (1991) SBT-11-0017

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Rat oral developmental study (SBT-00-0012)

Flumioxazin was administered orally *via* gavage to groups (22) pregnant female rats at concentrations of 0, 1, 3, 10 and 30 mg/kg/day from gestation day 6 to 15. Dams were culled on GD 20 and fetuses were removed by caesarean section and examined.

No maternal signs of toxicity were observed, with food consumption, body weights or clinical signs of toxicity in treated animals being comparable to the concurrent control group. The number of live fetuses ($p<0.05$) and fetal body weights ($p<0.01$) were significantly decreased in the high dose group, where as the number of *corpora lutea*, implantations and sex ratio were similar in all groups.

TABLE 23: EFFECTS ON MATERNAL ANIMALS

Parameter	Dose level (mg/kg/d)				
	0	1	3	10	30
Body weight gain (g) gestation day 6-20	95	96	101	97	85**
Necropsy Retention of dark reddish fluid in uterus Retention of dark reddish material in uterus	0/22 0/22	0/21 0/21	0/21 0/21	0/22 0/22	1/19 1/19

**: Statistically significance ($p<0.01$)

The changes of body weigh gain and necropsy in 30 mg/kg/d group were considered to be attributable to the foetal toxicity.

The incidence of fetuses with cardiovascular abnormalities, primarily ventricular septal defects (VSD), were increased in the 30 mg/kg/d groups, reaching statistical significance at the high dose group ($p<0.01$). Test material related increases (which reached statistical significance, $p<0.01$) for scapular curvature (malformation) and wavy ribs (minor anomaly), were observed. Test material related decreases in ossified sacrococcygeal vertebral bodies were also observed and which were considered to be related to the decreased fetal body weights, rather than due to a direct effect of the test material.

TABLE 24: SELECTED FETAL DATA FROM THE RAT ORAL DEVELOPMENTAL STUDY

Parameter	Dose level (mg/kg/d)				
	0	1	3	10	30
LITTER DATA					
Mean live fetuses (n)	14 ± 1.0	13 ± 2.6	14 ± 2.0	14 ± 2.1	11 ± 4.1*
Fetal body weights M /F (g)	3.51 / 3.34	3.53 / 3.39	3.53 / 3.34	3.54 / 3.34	3.00** / 2.79**
VISCERAL EXAMINATION					
No. examined (n) [M / F]	147 [67 / 80]	138 [73 / 65]	144 [76 / 68]	144 [64 / 80]	102 [54 / 48]
Cardiovascular abnormalities (n) [%]	8 [5.4%]	7 [5.1%]	10 [6.9%]	13 [9.0%]	36** [35.3%]
Historical control data for cardiovascular abnormalities 7.5%					
VSD (n) [%]	2 [1.4%]	1 [0.7%]	2 [1.4%]	6 [4.2%]	26** [25.5%]
SKELETAL EXAMINATION					
No. examined (n) [M / F]	154 [71 / 83]	144 [76 / 68]	152 [78 / 74]	153 [68 / 95]	111 [60 / 51]
Scapula curvature (n) [%]	0 [0.0%]	0 [0.0%]	0 [0.0%]	0 [0.0%]	10** [9.0%]
Wavy ribs (n) [%]	1 [0.6%]	1 [0.7%]	7 [4.6%]	0 [0.0%]	27** [24.3%]
Ossified sacrococcygeal vertebral bodies (n) (M/F)	8.9 ± 0.61 / 8.8 ± 0.62	9.0 ± 0.48 / 8.9 ± 0.49	9.1 ± 0.48 / 8.8 ± 0.62	9.0 ± 0.46 / 8.9 ± 0.34	8.5 ± 0.55** / 8.2 ± 0.44*

* $p<0.05$; ** $p<0.01$

Based on the result of this study, the NOAEL for developmental toxicity was considered to be 10 mg/kg/d, based on increased incidence of mortality, cardiac VSDs, wavy ribs, curvature of the scapular and reduced ossification of sacrococcygeal vertebral bodies, and decreased body weight in the fetuses of rats. The litter observation was considered to be related to the decrease in fetal body weights. No signs of maternal toxicity were observed, therefore the maternal NOAEL for considered to be greater than 30 mg/kg/d.

Rabbit oral developmental study (SBT-11-0017)

Flumioxazin was administered orally *via* gavage to groups of 20 pregnant female rabbits at concentrations of 0, 300, 1000 and 3000 mg/kg/day from gestation day 7 to 19. Dams were culled on GD 29 and fetuses were removed by caesarean section and examined.

Reductions in maternal body weight gains and relative and absolute food consumption were observed in the 3000 mg/kg/day group.

TABLE 25: EFFECTS ON MATERNAL ANIMALS

Parameter	Dose level (mg/kg/d)			
	0	300	1000	3000
Body weight gain (kg) gestation day 7-19	0.17	0.18	0.14	0.05*
Food consumption (g/d) gestation day 7-19	165.1	160.2	150.3	135.1*

*: Statistically significance ($p<0.05$)

No significant differences in pre or post implantation loss or early / late resorptions were observed. No test material related fetal changes were observed, with external / visceral and skeletal malformations / variations in test material treated animals being comparable to the concurrent control.

Based on the result of this study, the NOAEL for developmental toxicity was considered to be greater than 3000 mg/kg/d (the highest dose tested). The maternal NOAEL was considered to be 1000 mg/kg/d based on reductions in maternal body weight gains and relative and absolute food consumption.

4.11.2.2 DEVELOPMENTAL TOXICITY: DERMAL

The results of rat developmental studies are summarised in the following table:

TABLE 26: OVERVIEW OF EXPERIMENTAL STUDIES ON DEVELOPMENTAL TOXICITY AFTER DERMAL ADMINISTRATION

Method	Results	Remarks[1]	Reference
rat (SD) developmental study (24 females/gp) dermal: occluded (6 h/d) 0, 30, 100, 300 mg/kg/d (exposed from GD 6 – 15) EPA OPP 83-3; GLP	NOAEL maternal toxicity: >300 mg/kg/day based on no effects observed up to the highest dose tested NOAEL developmental toxicity: *ca*. 100 mg/kg/day based on increased incidence of cardiac ventricular septal defects, wavy ribs and reduced ossification of sacrococcygeal vertebral bodies	1 (reliable without restriction) **key study** **purity: 94.8%**	Kawamura (1991) SBT-10-0021

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Rat dermal developmental study (SBT-10-0021)

Flumioxazin was administered dermally to groups of 24 pregnant female rats at concentrations of 0, 30, 100 and 300 mg/kg/day from gestation day 6 to 15. Dams were culled on GD 20 and fetuses were removed by caesarean section and examined.

No maternal signs of toxicity were observed, with food consumption, body weights or clinical signs of toxicity in treated animals being comparable to the concurrent control group. The number of live fetuses ($p<0.01$) and fetal body weights (F: $p<0.05$) were significantly decreased in the high dose group, where as the number of *corpora lutea* and implantations were similar in all groups.

TABLE 27: EFFECTS ON MATERNAL ANIMALS

Parameter	Dose level (mg/kg/d)			
	0	30	100	300
Clinical sign Attachment of reddish material around vagina	1/23	0/22	2/22	4/22
Body weight gain (g) gestation day 6-20	99	90*	97	72**

**: Statistically significance ($p<0.01$)
The changes of clinical sign and body weigh gain in 300 mg/kg/d group were considered to be attributable to the foetal toxicity.

The incidence of fetuses with cardiovascular abnormalities, primarily VSD was significantly increased ($p<0.01$) in the 300 mg/kg/d group. Test material related increases in wavy ribs (minor anomaly; $p<0.01$)), were observed. In addition, test material related decreases in ossified sacrococcygeal vertebral bodies (statistically significant for females [$p<0.05$]) were also observed. This was considered to be related to the decreased fetal body weights, rather than due to a direct effect of the test material.

TABLE 28: SELECTED FETAL DATA FROM THE RAT DERMAL DEVELOPMENTAL STUDY

Parameter	Dose level (mg/kg/d)			
	0	30	100	300
LITTER DATA				
Mean live fetuses (n)	13.6 ± 2.41	12.2 ± 3.07	13.3 ± 2.36	8.1± 5.90**
Fetal body weights M/F (g)	3.38 / 3.24	3.51 ** / 3.33	3.43 / 3.24	3.10 * / 3.04
Total no. of embryonic deaths (%)	20 (6.0%)	16 (5.6%)	20 (6.4%)	122 (40.7%)**
VISCERAL EXAMINATION				
No. examined (n) [M/F]	153 [75 / 78]	133 [58 / 75]	144 [75 / 69]	88 [53 / 35]
Cardiovascular abnormalities (n) [%]	5 (3.3%)	4 (3.0%)	10 (6.9%)	19 (21.6%)**
Historical control data for cardiovascular abnormalities 7.5%				
VSD (n) [%]	1 (0.7%)	1 (0.8%)	2 (1.4%)	13 (14.8%)**
SKELETAL EXAMINATION				
No. examined (n) [M / F]	159 [81 / 78]	136 [62 / 74]	149 [77 / 72]	90 [57 / 33]
Wavy ribs (n) [%]	0 (0.0%)	0 (0.0%)	2 (1.3%)	18 (20.0%)
Ossified sacrococcygeal vertebral bodies (n) (M/F)	8.8 ± 0.41 / 8.7 ± 0.37	9.0 ± 0.37 / 8.8 ± 0.45	8.9 ± 0.66 / 8.6 ± 0.55	8.5 ± 0.78 / 8.4 ± 0.50*

* $p<0.05$; ** $p<0.01$

Based on the results of this study, the NOAEL for developmental toxicity was considered to be 100 mg/kg/d, based on increased incidence of VSD, wavy ribs and reduced ossification of sacrococcygeal vertebral bodies in the fetuses of rats. The latter observation was considered to

be related to the decrease in fetal body weights. No signs of maternal toxicity were observed, therefore the maternal NOAEL for considered to be greater than 300 mg/kg/d.

4.11.3 SUMMARY AND DISCUSSION OF REPRODUCTIVE TOXICITY

Summary of the Dossier submitter's proposal

The substance has a harmonised classification for reproductive toxicity in category 1B based on teratology studies (consistent with guideline EPA OPP 83-3) in SD rats (oral and dermal) and it is not contested in the dossier, that these results are severe enough to warrant classification. Since the time the current classification was decided, new data have been generated leading the DS to suggest the removal of the classification as toxic to reproduction. The new data consisted of a negative teratology study in NZW rabbits (guideline EPA OPP 83-3) and a large set of mechanistic data. The DS argued that the effects seen in rats are not relevant for humans.

The principal argument does not question that flumioxazin causes significant developmental toxicity/teratogenicity in rats but rather that there is a clear species difference with respect to susceptibility to the specific mechanism, with rats more sensitive than humans which are in turn more sensitive than rabbits.

The database contains the following;

1. **The original guideline data** set consisting of a 2-generation study in rats, oral and dermal developmental toxicity studies in rats and a developmental toxicity study in rabbits.

2. **The original data set of mechanistic studies** with flumioxazin:
   -Haematotoxicity of flumioxazin
   -Placental transfer
   -Critical period of embryonic sensitivity
   -Histopathological study of early stages of development in rat and rabbits foetuses following exposure to flumioxazin
   -Pathogenesis of developmental effects of flumioxazin
   -Studies of PPO inhibition/PPIX accumulation in embryos
   -Species differences in PPIX accumulation
   -Critical period for PPIX accumulation in rat and rabbit embryos

3. **Recent mechanistic studies**
   -Pharmacokinetics rat/rabbit
   -Chronological changes of morphology and population of circulating erythroblasts in rat embryos during yolk sac haematopoiesis.
   -Inhibition of PPO activity by flumioxazin and its major metabolites, 3-OH S-53482, 4-OH S-53482 and APF in rat liver mitochondria.
   - Species differences in PPIX accumulation induced by flumioxazin in cryopreserved hepatocytes among rat, rabbit, monkey and human.
   - Effects of flumioxazin and metabolites of flumioxazin on haem synthesis pathway and cell proliferation in K562 cells.
   -Physiologically based pharmacokinetic (PBPK) modelling of flumioxazin in rats and humans and *in silico*.

Summary of guideline Developmental toxicity studies.

Flumioxazin induced embryolethality and teratogenicity in the rat following dosing *via* both the oral (at 30 mg/kg bw/day) and dermal (at 300 mg/kg bw/day) routes. Abnormalities mainly consisted of cardiac ventral septal defect (VSD). In addition, there was an increase in the

incidence of wavy ribs and reduced ossification of sacrococcygeal vertebral bodies Furthermore, foetal growth retardation was also observed in both studies. This observation was supported by the occurrence of reduced litter size (embryofoetal lethality) and reduced pup weight seen in the 2-generation study. These effects were observed in the rat at relatively low levels and in the absence of maternal toxicity. In contrast, flumioxazin showed no evidence of developmental toxicity in the rabbit even in the presence of maternal toxicity. The maximum dose administered in the rabbit study was 100-fold greater (3000 mg/kg/d) than the maximum dose administered in the rat oral developmental study.
In addition, developmental toxicity studies were conducted with two closely related structural analogues of flumioxazin. S-23031 was shown to be negative for developmental toxicity in both the rat and rabbit. S-23121 was shown to cause increased incidence of cardiac VSD, growth retardation and embryo lethality in the absence of signs of maternal toxicity in the rat, and had no adverse effect on development up to doses causing maternal toxicity in the rabbit.

Proposed mechanism of action.

Flumioxazin is a herbicide which disrupts photosynthesis probably by inhibition of PPO and auto-oxidation of protoporphyrinogen IX (PPPIX) to protoporphyrin IX (PPIX). Porphyrin biosynthesis is common to plants and animals, as part of chlorophyll and as part of the penultimate enzyme in haem synthesis, respectively. The mode of action of flumioxazin and N-phenylimide herbicides is presented in Figure 1.

The mechanism by which developmental toxicity is produced by flumioxazin is presented in Figure 2 and is postulated as follows: flumioxazin inhibits a key enzyme, (PPO) in rats, interfering with normal haem synthesis in the mitochondria. Inhibition of PPO leads to an accumulation of its substrate, PPPIX in the mitochondrion. The accumulated PPPIX leaves the mitochondrion and undergoes non-enzymatic oxidation to PPIX in the plasma. The resulting PPIX is out of reach of the final enzyme in haem synthesis (ferrochelatase) and cannot be transferred to haem, resulting in anaemia. The foetal anaemia leads to hypoxia in foetal tissues followed by suppressed liver function and a decrease in protein synthesis. This decreased protein synthesis would result in wavy ribs and changes in osmotic forces are thought to be responsible for the oedema observed in the foetus. Concurrently, the foetus may compensate for the anaemia by pumping a greater volume of blood leading to the observed enlargement of the heart just prior to closure of the interventricular foramen, thus resulting in the delayed closure of the foramen and VSD. Thus, the VSD observed in teratology studies is considered to be produced by mechanical distortion of the heart. The two other signs of developmental toxicity reported (growth retardation and foetal death) are also considered related to the hypoxia produced by the anaemic condition in the foetus.

Flumioxazin induced embryolethality and teratogenicity in the rat following dosing *via* both the oral and dermal routes. Evidence of teratogenicity was exhibited in the form of cardiovascular abnormalities, mainly VSD, and an increase in the incidence of wavy ribs was also observed. Furthermore, fetal growth retardation was also observed in both studies. These effects observed in the rat were in the absence of maternal toxicity. In contrast to the rat, flumioxazin showed no evidence of developmental toxicity in the rabbit even in the presence of maternal toxicity. The maximum dose administered in the rabbit study was 100-fold greater (3000 mg/kg/d) than the maximum dose administered in the rat oral developmental study.

In the multi-generation study there was an increase in resorptions and a decrease in pup survival and average pup weight which were probably seen as an extension of the causal effects which resulted in the increased embryolethality and growth retardation observed in the rat developmental studies. No treatment related effects on fertility were observed.

4.12 MECHANISTIC STUDIES

The results of mechanistic studies are summarised in this section. New mechanistic studies which were conducted after the publication of flumioxazin in the 28th ATP of the DSD are summarised first (Section 4.12.1), whilst the previous mechanistic studies which established the mode of action for teratogenicity in the rat are summarised in Sections 4.12.2 to 4.12.4.

4.12.1 NEW MECHANISTIC STUDIES

TABLE 29: OVERVIEW OF NEW MECHANISTIC STUDIES CONDUCTED ON FLUMIOXAZIN

Method	Results	Remarks[1]	Reference
rat pharmacokinetic study (3 females/gp) oral: gavage rat (Crl:CD (SD)): 1000 mg/kg/ 3.7 MBq/single dose No guideline available; non-GLP	The total amounts of ^{14}C excreted into bile and urine and ^{14}C remained in the carcass showed that the absorption (bile + urine + carcass) in females was 12.3% after a single oral administration of flumioxazin at 1000 mg/kg.	2 (reliable with restrictions) **key study** **[phenyl-U ^{14}C]flumioxazin purity: 98.6%** **Flumioxazin**	Takaku, T. (2012a) SBM-0092
rat / rabbit pharmacokinetic study (4 females/gp) oral: gavage rat (HW): 30 mg/11.3 MBq/5 mL/kg/d rabbit (NZW): 30 mg/1.12 MBq/0.5 mL/kg/d (exposure from GD 6 – 12) Japanese guidelines on non-clinical pharmacokinetic studies no.496; non-GLP	In rats significantly higher transfer of radioactivity to blood cells was observed compared with rabbits. Elimination of radioactivity from female reproductive tissue of both species was slower than that from plasma, with only a small amount of radioactivity being transferred to the fetus.	2 (reliable with restrictions) **key study** **[phenyl-U ^{14}C]flumioxazin purity: 99.9%** **Flumioxazin purity: 99.4%**	Shirai, N. (2009) SBM-0081
development of rat erythroblasts in rat embryos, *ex vivo* rat (SD) male/female No test material added, study used to examine differentiation of developing erythrocytes No guideline available; non-GLP	In rats differentiation of circulating erythroblasts in rat embryos from embryonic day 11 to 14 was synchronised.	2 (reliable with restrictions) **key study** **purity: n/a**	Ihara, R. (2011) SBT-0117
inhibitory action against the enzyme PPO obtained from rat liver mitochondria, *in vitro* S-53482: 10 pM - 1 µM 3-OH S-53482: 100 pM - 10 µM 4-OH S-53482: 1 nM - 100 µM APF: 1 nM - 100 µM No guideline available; non-GLP	Flumioxazin has the strongest inhibitory activity among the 4 substances tested, followed by 3-OH S-53482 and 4-OH S-54382, which were 13.7 and 147 times weaker than flumioxazin. APF does not have any inhibitory activity against PPO up to 100 µM.	2 (reliable with restrictions) **key study** **Flumioxazin purity: 99.4%** **3OH S-53482 purity: 99.6%** **4OH S-53482 purity: 99.0%** **APF purity: 99.9%**	Abe, J. (2011a) SBT-0118
K562 cell differentiation into erythroid cells in the presence of flumioxazin, *in vitro*	PPIX accumulation in K562 cells was observed at concentrations of 1 µM and greater in dose dependent manner, there however was no effect	2 (reliable with restrictions)	Kawamura, S. (2012a)

Method	Results	Remarks[1]	Reference
0.01 - 5 μM No guideline available; non-GLP	on cell proliferation or haem synthesis at the highest dose tested.	**key study** **purity: not stated**	SBT-0119
K562 cell differentiation into erythroid cells in the presence of metabolites of flumioxazin, *in vitro* 5 μM No guideline available; non-GLP	There was no effect on protoporphyrin IX content, heme synthesis and cell proliferation when K562 cells were treated with the metabolites, while flumioxazin increased protoporphyrin IX in K562 cells.	2 (reliable with restrictions) **key study** **purity: not stated**	Kawamura, S. (2012b) SBT-0123
species difference in accumulation of PPIX in primary hepatocytes from rat, rabbit, monkey & human, *in vitro* 0.01 - 0.3 μg/mL No guideline available; non-GLP	The induction ratios of PPIX following treatment with flumioxazin at 0.3 μg/mL were 10.3, 1.1, 1.4 and 4.4-fold in primary hepatocytes of rat, rabbit, monkey and human respectively. These results suggest that rat hepatocytes are more sensitive to flumioxazin treatment than the other 3 species, including human.	2 (reliable with restrictions) **key study** **purity: 99.4%**	Abe, J. (2011b) SBT-0120
PBPK modelling of flumioxazin in rats and humans, *in vitro* and *in silico* 5.6 – 100 μM No guideline available; non-GLP	The developed human PBPK model demonstrated that the human fetal exposure to flumioxazin following a maternal oral dose of 1000 mg/kg would be 0.68 ppm (1.92 μM), elucidating that exposure to flumioxazin in a human fetus would be relatively low, even at 1000 mg/kg.	2 (reliable with restrictions) **key study** **[phenyl-U ^{14}C]flumioxazin** **purity: 98.6%**	Takaku, T. (2012b) SBM-0093

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Biliary excretion study in female rats (SBM-0092)

The biliary excretion of [phenyl-U-^{14}C] flumioxazin was investigated in bile duct-cannulated female rats at a dose of 1000 mg/kg bw. Three female rats were orally dosed with radiolabelled flumioxazin by gavage, and bile, urine and faeces were collected at approximately 0-24, 24-48 and 48-72 hours after dosing. The gastrointestinal tract (with contents) was also collected from each animal at time of sacrifice. Total radioactive residues were determined in tissue samples and in the residual carcass. Most of the radioactivity (84.7%) was eliminated in the faeces by 72 hours after dosing. Excretion in urine and bile accounted for 6.8 and 5.2% of the dose, respectively, with very low amounts remaining in the gastrointestinal tract (0.6%) and the residual carcass (0.3%). Total ^{14}C recoveries accounted for 97.6% of the dose. The oral absorption of flumioxazin at 1000 mg/kg bw was calculated to be 12.3% by summing the urinary and biliary ^{14}C excretion and the ^{14}C remaining in the carcass.

Rat / rabbit pharmacokinetic study (SBM-0081)

Non-fasted pregnant rats and pregnant rabbits were administered ^{14}C-flumioxazin at 30 mg/kg/d for 7-days *via* oral gavage from gestation day 6 to 12. Concentrations of radioactivity in blood and plasma, excretion in urine and faeces, tissue concentrations, tissue distribution and transfer to female reproductive tissues were investigated. Composition of flumioxazin and its metabolites in urine, faeces, plasma, blood cells, liver, amniotic fluid and fetus were also examined.

Blood, plasma and excreta were obtained daily post dosing. In order to complete a time course, blood and plasma were also collected up to 24 hr post the final administration. Tissues were also

collected at 7 and 24hrs (rats) or 3 and 24hrs (rabbits) post the final dose. Tissues collected included kidney, liver, spleen, fat, ovaries, placenta, uterus and fetus along with amniotic fluid to determine concentration of radioactivity. Concentrations of flumioxazin and its metabolites were determined in urine, faeces, plasma, blood cells, liver, fetus and amniotic fluid.

Flumioxazin was rapidly excreted in pregnant rabbits (urine and faeces) and pregnant rats (mainly faeces). The concentration of radioactivity in blood and plasma almost reached steady state on day 4 and on day 2 for rats, respectively, and the concentrations in both blood and plasma of rabbits did not reach steady state within the 7 day dosing period. In rats flumioxazin was distributed at higher concentration in all tissues, except for the uterus, amniotic fluid and fetus. In rabbits flumioxazin was distributed at lower concentrations in all tissues, except for liver and kidney than in plasma. Elimination of radioactivity from female reproductive tissue of both species was slower than that from plasma, with only a small amount of radioactivity being transferred to the fetus.

Study examining chronological changes of morphology and population of circulating erythroblasts (SBT-0117)

Differentiation of circulating erythroblasts in rat embryos was investigated from embryonic development day (EDD) 11 through to EDD 14. Timed pregnant females were anaesthetised in the morning of the scheduled necropsy, uteri and embryos were removed. Embryos were exsanguinated from severed umbilical cord and embryonic blood cells from the same litter were pooled.

On day 11 of embryonic development more than 95% of circulating erythroblasts were composed of basophilic erythroblasts. From day 12 to 13 of embryonic development the predominant cell population was the polychromatophilic erythroblast (PCE). At day 14 the population of PCE was markedly reduced, with the orthochromatophilic erythroblast population becoming predominant.

The results of this study show that differentiation of circulating erythroblasts in rat embryos from embryonic Day 11 to 14 was synchronized.

Study examining the inhibition of PPO activity by flumioxazin and its major metabolites (SBT-0118)

Potential inhibitory enzyme activity of flumioxazin (S-53482; at 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM) and its major metabolites, 3-OH S-53482 (at 100 pM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM), 4-OH S-53482 (at 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM) and APF (at 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM) against protoporphyrinogen oxidase (PPO) was conducted *in vitro* using rat liver mitochondrial fraction.

In conclusion, flumioxazin has the strongest inhibitory activity among the 4 substances tested, followed by 3-OH S-53482 and 4-OH S-54382, which were 13.7 and 147 times weaker than flumioxazin. APF does not have any inhibitory activity against PPO up to 100 μM.

Study examining the effects of flumioxazin on the haem synthetic pathway and cell proliferation (SBT-0119)

The objective of this study was to investigate the effect of flumioxazin on the haem synthetic pathway in human erythroid cells, whereby K562 cells were induced to differentiate into erythroid cells using sodium butyrate (NaB), followed by treatment with flumioxazin at concentrations of ranging from 0.01 to 5 μM.

K562 cells were plated at 1 x 10^5 cells/mL/well (24-well plate) cells in RPMI medium containing flumioxazin at concentrations ranging from 0.01 to 5 μM. In order to stimulate differentiation, NaB was added to RPMI medium containing flumioxazin. Therefore, the concentration range of flumioxazin used was examined in the presence of NaB at all concentrations, 0, 0.01, 0.1, 1 and 5 μM. Cell densities were determined on days 2, 4, 6 and 8 (following sub-culturing at Day 4) with PPIX and haem content determined at the same intervals using LC/MS detection.

In conclusion, PPIX accumulation in K562 cells at 1.0 μM and above was observed in a dose dependent manner, with no effect on cell proliferation or haem synthesis up to the highest dose of 5.0 μM.

Study examining the effects of metabolites of flumioxazin on the haem synthetic pathway and cell proliferation (SBT-0123)

The objective of this study was to investigate the effects of metabolites of flumioxazin on the haem synthetic pathway in human erythroid cells, whereby K562 cells were induced to differentiate into erythroid cells using sodium butyrate (NaB), followed by treatment with flumioxazin or each of three metabolites (3OH-flumioxazin, 4OH-flumioxazin and APF) at a concentration of 5 μM.

K562 cells were plated onto 6 cm dishes at 5 x 10^5 cells/5mL/dish in RPMI medium containing flumioxazin or the metabolites at a concentration of 5 μM. In order to stimulate differentiation, NaB was added to RPMI medium containing flumioxazin or the metabolites. Therefore, the concentration range of flumioxazin or the metabolites used were examined in the presence of NaB at two different concentrations, 0 and 5 μM (flumioxazin, 3OH-flumioxazin, 4OH-flumioxazin and APF). Cell densities were determined on days 2, 4, 6 and 8 (following sub-culturing at Day 4) with PPIX and haem content determined at the same intervals using LC/MS detection.

There was no effect on PPIX content, heme synthesis and cell proliferation when K562 cells were treated with the metabolites of flumioxazin. PPIX accumulation in K562 cells was observed with flumioxazin but there was no effect on cell proliferation or haem synthesis (consistent with the results of SBT-0119).

Study examining the species differences in accumulation of PPIX in primary hepatocytes SBT-0120)

The objective of this study was to investigate the species difference in the accumulation of PPIX due to the inhibition of protoporphyrinogen oxidase by flumioxazin *in vitro* using cyropreserved primary hepatocytes from rat, rabbit, monkey and human.

Cells were thawed and seeded at 0.5 x 10^6 cells/mL in a 24 well plate for approximately 24 hours. Flumioxazin was dissolved in DMSO and added to at final concentrations of 0, 0.01, 0.03, 0.1 and 0.3 μg/mL. No less than six replicates of each concentration were prepared, with each experiment conducted in triplicate for each lot of hepatocytes.

Following exposure to flumioxazin for 24 hours medium was removed, cells harvested and processed for potential PPIX accumulation using LC/MS analysis. The concentration of PPIX was expressed in terms of protein concentration.

Basal PPIX concentration in rat hepatocytes was 293 pg/mg protein and was increased by the addition of flumioxazin dose dependently, with a ~10-fold increase above the basal level when cells were exposed to 0.3 μg/mL of flumioxazin.

Basal PPIX concentration in rabbit hepatocytes was similar to that in the rat, however PPIX concentration was not increased in rabbit hepatocytes when exposed to flumioxazin. A similar effect was seen in monkey hepatocytes, whilst the basal PPIX concentration was much lower (42 pg/mg protein), no accumulation of PPIX was observed when monkey hepatocytes were exposed to flumioxazin.

In all 3 batches of human hepatocytes, basal PPIX levels were much lower than that of the rat and rabbit, and the mean PPIX concentration of the 3 lots in the control samples was 180 pg/mg protein. An approximate 4.4 fold increase in PPIX concentration was observed following exposure to flumioxazin up to 0.3 μg/mL.

In conclusion, the induction ratios of PPIX following treatment with flumioxazin at 0.3 μg/mL were 10.3, 1.1, 1.4 and 4.4 fold in primary hepatocytes of rat, rabbit, monkey and human respectively. These results suggest that the rat hepatocytes are more sensitive to flumioxazin treatment than the other 3 species, including humans.

PBPK modelling of flumioxazin in rats and humans (SBM-0093)

The objective of this study was to develop a physiologically based pharmacokinetic (PBPK) model for flumioxazin in order to predict parent flumioxazin concentrations in blood and fetus of pregnant humans based on data obtained in the rat. An *in vitro* metabolism study using rat and human liver microsomes was carried out to determine any species differences in the metabolism of flumioxazin between rat and human. Physiological data for humans were cited from the literature and the human model was developed to predict the pharmacokinetics in humans in several tissues. Whilst it is impossible to measure experimentally human fetal concentrations of flumioxazin, development of the PBPK model in the pregnant rat was scaled to humans to provide an estimate of the disposition of flumioxazin in pregnant humans.

The metabolites produced by human microsomes were nearly identical to those produced by female rat liver microsomes, it was therefore concluded that there was no species difference in the metabolism of [phenyl-U-^{14}C]flumioxazin.

The rat PBPK model accurately simulates the blood, liver and fetus concentrations of flumioxazin at single oral doses of 30 mg/kg. The rat model was extrapolated to the pregnant human based on human physiological data and the *in vitro* metabolism data. The fraction of dose absorbed at 1000 mg/kg in the rat was 12% (see SBM-0092). These values were then combined to develop the PBPK model at a dose of 1000 mg/kg.

Simulated blood and fetus concentrations of flumioxazin at an oral dose of 1000 mg/kg in pregnant humans were estimated at 0.86 ppm and 0.68 ppm (maximum concentration), respectively, elucidating that exposure to flumioxazin in the pregnant human and fetus would be relatively low, even at 1000 mg/kg.

The developed human pregnant PBPK model demonstrates that the human fetal exposure to flumioxazin following a maternal oral dose of 1000 mg/kg would be 0.68 ppm (1.92 μM).

4.12.2 HAEMATOTOXICITY AND PLACENTAL TRANSFER MECHANISTIC STUDIES

TABLE 30: OVERVIEW OF HAEMATOXICITY AND PLACENTAL TRANSFER MECHANISTIC STUDIES CONDUCTED ON FLUMIOXAZIN

Method	Results	Remarks[1]	Reference
rat (SD) study examining the mechanism of haematotoxicity (up to 30 animals/sex/gp) oral: feed study 1: 0, 3000, 10000 ppm study 2: 0, 3000 ppm (exposure: study 1: 37 days; study 2: 15 days) No guideline available; non-GLP	Flumioxazin induced anaemia in rats can be classified as sideroblastic anaemia resulting primarily from the defective haem pathway during the process of haemoglobin biosynthesis considering the increase in porphyrins and siderocytes. The increased blood porphyrin level suggested the S-53482 induces porphyria in rats.	2 (reliable with restrictions) **key study** **purity: 94.8%**	Yoshida (1996) SBT-0059
rat / mouse (SD / ICR) study examining placental transfer of flumioxazin (24 animals/gp) oral: gavage 30 mg/kg (exposure:GD12) EPA OPP 85-1, non-GLP	In mice significantly higher transfer of radioactivity to blood cells was observed compared with rats. Elimination of radioactivity from female reproductive tissue of both species was slower than that from blood (blood cell & plasma), with only a small amount of radioactivity being transferred to the fetus.	1 (reliable without restriction) **key study** **[phenyl-U ^{14}C]flumioxazin** **purity: >99%**	Isobe (1992) SBM-20-0015
rat / rabbit (SD / JW) study examining placental transfer of flumioxazin oral: gavage 30 mg/kg (exposure: rats GD 12 / mice GD10) EPA OPP 85-1, non-GLP	In rats significantly higher transfer of radioactivity to blood cells was observed compared with rabbits. Elimination of radioactivity from female reproductive tissue of both species reached maxima 2-4 hrs after administration and decreased rapidly thereafter, with only a small amount of radioactivity being transferred to the fetus.	2 (reliable without restriction) **key study** **[phenyl-U ^{14}C]-flumioxazin** **purity: >99%**	Isobe (1993) SBM-30-0032

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Study examining the mechanism of haematotoxicity in the rat (SBT-0059)

In a 15 and 37 day study S-53482 was fed to rats (24 or 30 animals/sex/group) at doses of 0, 3,000 and 10,000 ppm. The purpose of the study was to determine the effects on the haematological system to explain the mechanism by which the test material induces anaemia. Haematological parameters were assessed by examining the erythrocyte count, reticulate count, neutrophil count, haemoglobin concentration, haematocrit value, bone-marrow myeloid/ erythroid (M/E) ratio, MCV, MCH and MCHC. Additional urinary coproporphyrin and biochemistry were evaluated. During the treatment period, no animal died, nor did body weights or food consumption show any consistent remarkable changes.

The haematological changes at 3,000 ppm and higher dose groups included decreases in erythrocyte count, haemoglobin concentration and haematocrit value with decreases occurring progressively over time from day 5 through day 15.

The neutrophils and reticulocytes decreased during early treatment but increased thereafter, and the bone-marrow M/E ratio tended to increase slightly on day 2 but then decreased. These

observations suggest that erythropoiesis was depressed during the early treatment period but subsequently was stimulated.

In contrast MCV, MCH and MCHC were decreased from days 15 through day 37. These decreases suggest the persistence of a haemoglobin deficiency in erythrocytes even after increased erythropoiesis and the generation of hypochromic, microcytic erythrocytes.

In the blood biochemistry tests, the abnormal findings in treated groups included increases in serum iron, total cholesterol, blood urea nitrogen, sodium & potassium as well as decreases in GOT, uric acid, calcium and triglyceride. Increased liver and spleen weights were also observed in the treated groups. The urinary coproporphyrin and erythrocyte protoporphyrin levels were increased in the 3,000ppm group.

There was an increase in the number of siderocytes in the peripheral blood and the concentration of porphyrin in urine and erythrocytes increased with time. The accumulation of porphyrins in the body signifies that porphyrin is not converted into haemoglobin, and the increase in siderocytes indicates that due to defective haemoglobin synthesis, iron accumulated in excess erythrocytes.

In conclusion, S-53482 (flumioxazin) induced anaemia in rats can be classified as sideroblastic anaemia resulting primarily from the defective haem pathway during the process of haemoglobin biosynthesis considering the increase in porphyrins and siderocytes. The increased blood porphyrin level suggested the S-53482 induces porphyria in rats.

Study examining the placental transfer in rats and mice (SBM-20-0015)

Non-fasted pregnant rats and pregnant mice were administered ^{14}C-flumioxazin at 30 mg/kg once by oral gavage from gestation day 12 for rats and day 10 of gestation for mice to determine the extent of fetal exposure to flumioxazin and its metabolites.

Blood, blood cells and plasma were collected at sacrifice, 1, 2, 4, 8, 24 and 72 hrs after administration. Tissues were also collected at this time included bone marrow, fat, fetus, kidney, liver, ovary, placenta, spleen and uterus to determine the concentration of radioactivity. Concentrations of flumioxazin and its metabolites were determined in urine, faeces, blood, blood cells, plasma, liver and fetus (mice only).

Flumioxazin was rapidly excreted in pregnant rats and mice (mainly faeces), with excretion being more rapid in mice than rats. ^{14}C-concentrations in most of the analysed tissues of mice reached maxima and decreased more rapidly than those of rats. In both rats and mice flumioxazin was distributed at higher concentrations in the blood. Elimination of radioactivity from female reproductive tissue of both species was slower than from plasma, with only low amounts of radioactivity being transferred to the fetus. The maximum ^{14}C concentration in the fetus was 1.05 and 1.72 ppm for rats and mice, respectively.

The metabolism of flumioxazin was qualitatively the same between pregnant rats and mice. However, it was suggested that 3-hydroxylation activity might be higher in pregnant rats than in pregnant mice. The major metabolite in fetus of mice at 1 hr after administration was 4-OH-flumioxazin.

Study examining the placental transfer in rats and rabbits (SBM-30-0032)

Non-fasted pregnant rats and pregnant rabbits were administered ^{14}C-flumioxazin at 30 mg/kg once by oral gavage from gestation day 12 to determine the extent of fetal exposure to flumioxazin and its metabolites.

Blood, blood cells and plasma were collected at sacrifice, 1, 2, 4 and 24 hrs after administration. Tissues were also collected at this time included bone marrow, fat, fetus, kidney, liver, ovary, placenta, spleen and uterus to determine the concentration of radioactivity. Concentrations of flumioxazin and its metabolites were determined in urine, faeces, blood cells, plasma, liver and fetus.

Flumioxazin was excreted in pregnant rats and rabbits (mainly faeces), with excretion being more rapid in rats than rabbits. ^{14}C-concentrations in most of the analysed tissues of rats and rabbits including the fetus reached maxima at 2-4 hrs, although concentrations in rats were much higher and decreased more rapidly than in rabbits. In both rats and rabbits flumioxazin was distributed at higher concentrations in the liver and kidney compared with other tissues. Maximum ^{14}C concentrations in maternal tissues were higher than those observed in the fetus or amniotic fluid in both species. For those time points, where sufficient fetal tissue was available for metabolite identification (1 and 24 hours for rats and 2 and 24 hours for rabbits), the highest concentration of parent flumioxazin was 0.06 (at 1 hour after dosing) and 0.02 (at 2 hours after dosing) µg/g tissue for rats and rabbits, respectively. These data indicate that flumioxazin crosses the placenta and that both parent and various metabolites are present in measurable quantities in the fetus. At the same dose level, the concentration of radioactivity and of flumioxazin is greater in rat foetuses than rabbit foetuses but less than found in the mouse. No clear pattern of absorption, distribution, metabolism or excretion was evident which could account for the species-specific developmental toxicity in rats.

4.12.3 FURTHER DEVELOPMENTAL MECHANISTIC STUDIES

TABLE 31: OVERVIEW OF FURTHER DEVELOPMENTAL MECHANISTIC STUDIES CONDUCTED ON FLUMIOXAZIN AND ITS METABOLITES

Method	Results	Remarks[1]	Reference
rat (SD) study examining the critical period for developmental toxicity (5 females/gp) oral: gavage 400 mg/kg (exposure: single dose on GD 11, 12, 13, 14 or 15) EPA OPP 83-1, non-GLP	The data confirmed that the most sensitive developmental stage common to VSD, embryonic mortality and reduced fetal body weight was GD 12. Since all 3 endpoints peaked in incidence on GD 12, it is suggested that the mechanism involved in all 3 parameters is common to teratogenicity, embryolethality and growth retardation.	2 (reliable with restrictions) **key study** **purity: 94.8%**	Kawamura (1993a) SBT-30-0044
rat (Wistar) study examining the effects of irradiation on embryonic development irradiation: direct exposure 25, 50, 100, 200, 400r (single exposure GD 9) No guideline available; non-GLP	Direct exposure of developing embryos on GD 9 at increasing doses of irradiation was associated with increased incidences of cardiovascular abnormalities, which were associated with the high rate or prenatal mortality.	2 (reliable with restrictions) **supporting study** **purity:n/a**	Wilson *et al* (1953)
rat (Donryu) study examining the effects of nimustine hydrochloride on cardiovascular embryonic development intraperitoneal injection 10, 11, 13 mg/kg (single exposure GD 7, 8 or 9) No guideline available; non-GLP	Rates of resorption tended to be higher in accordance with increasing dosage. The highest frequency of cardiovascular anomalies was found in the groups treated on GD 8, but there was no difference in the rates induced by the three dosages of nimustine hydrochloride administered.	2 (reliable with restrictions) **supporting study** **purity: not stated**	Miyagawa *et al* (1988)

Method	Results	Remarks[1]	Reference
rat / rabbit (SD / JW) study examining the histopathological effects of flumioxazin on embryonic development oral: gavage 0, 1000 mg/kg (single exposure GD 12) EPA OPP 83-3; non-GLP	Flumioxazin did not induce VSD due to direct injurious effect on embryonic heart tissue. The effects were likely attributed to an indirect mechanism, where flumioxazin inhibits PPO in rat embryos only, thereby interfering with normal haem biosynthesis resulting in embryonic anaemia. The embryo compensates for the anaemic hypoxia by increasing heart stroke volume, leading to hypertrophy of the heart. VSD defects result from mechanical distortion of the heart	2 (reliable with restrictions) **key study** **purity: 94.8%**	Kawamura & Yoshioka (1997) SBT-0064 and Kawamura (1993b) SBT-30-0043
rat (SD) study examining the pathogenesis of developmental effects produced by flumioxazin oral: gavage 0, 400 mg/kg (single exposure GD 12) EPA OPP 83-3; non-GLP	Data from this study suggest that the enlarged heart, oedema and anaemia preceding the occurrence of fetal mortality may be instrumental in the cause of death. Similarly, the occurrence of enlarged heart preceding the failure of the interventricular closure would be related to the pathogenesis of this finding.	2 (reliable with restrictions) **key study** **purity: 94.8%**	Kawamura (1997) SBT-0065
rat (SD) developmental study (21 females/gp) oral: gavage 0, 1, 3, 10, 20 mg/kg/d (exposed GD 6 – 15) EPA OPP 83-3; GLP	NOAEL maternal toxicity: >20 mg/kg/day based on no signs of maternal toxicity observed up to the highest dose tested NOAEL developmental toxicity: *ca*. 10 mg/kg/day based on increased incidence of cardiac ventricular septal defects, growth retardation and embryo lethality	1 (reliable without restriction) **key study** **S-23121** **purity: 94.7%**	Kawamura (1990b) PPT-00-0023
rabbit (NZW) developmental study (20 females/group) oral: gavage 0, 2, 4, 8, 15 mg/kg/d (exposed GD 7 – 19) EPA OPP 83-3; GLP	NOAEL maternal toxicity: *ca*. 2 mg/kg/day based on reductions in maternal body weight / body weight gain and relative / absolute food consumption NOAEL developmental toxicity: >15mg/kg/day based no effects observed up to the highest dose tested	1 (reliable without restriction) **key study** **S-23121** **purity: 94.7%**	Hoberman (1990) PPT-01-0020
rat (SD) developmental study (25 females/group) oral: gavage 0, 50, 500, 1500 mg/kg/d (exposed GD 6 – 15) EPA OPP 83-3; GLP	NOAEL maternal toxicity: >1500 mg/kg/day based on no signs of maternal toxicity observed NOAEL developmental toxicity: >1500mg/kg/day based on no effects observed up to the highest dose tested	1 (reliable without restriction) **key study** **S-23031** **purity: 94.4%**	Lemen (1991a) SAT-11-0024
rabbit (NZW) developmental study (17 females/group) oral: gavage 0, 100, 200, 400, 800 mg/kg/d (exposed GD 7 – 19) EPA OPP 83-3; GLP	NOAEL maternal toxicity: *ca*. 400 mg/kg/day based on reductions in maternal body weight / body weight gain and mortality NOAEL developmental toxicity: >800mg/kg/day based on no effects observed up to the highest dose tested	1 (reliable without restriction) **key study** **S-23031** **purity: 94.4%**	Lemen (1991b) SAT-11-0025

1.Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Study examining the critical period for developmental toxicity in rats (SBT-30-0044)

Pregnant animals (5/gp) received a single oral administration of flumioxazin (400 mg/kg) on either GD 11, 12, 13, 14 or 15 to determine the critical period of embryonic sensitivity. All dams were sacrificed on GD 20. Live fetuses were fixed and examined for VSD.

The data confirmed that the most sensitive developmental stage common to VSD, embryonic mortality and reduced fetal body weight was GD 12. Since all 3 endpoints peaked in incidence on GD 12, it is suggested that the mechanism involved in all 3 parameters is common to teratogenicity, embryolethality and growth retardation.

TABLE 32: INCIDENCES OF EMBRYONIC DEATHS, FETAL BODY WEIGHTS AND VENTRICULAR SEPTAL DEFECTS

Parameter	Time after administration (h)				
	11	12	13	14	15
Embryonic mortality	2.7%	39.4%	16.1%	9.9%	6.3%
Male fetal body weight	3.34g	3.23g	3.73g	3.59g	3.67g
Female fetal body weights	3.22g	2.95g	3.49g	3.14g	3.46g
VSD	6.9%	14.0%	5.8%	4.7%	2.2%

Both x-ray irradiation (Wilson *et al.* (1953) and nimustine (Miyagawa *et al.* (1988) produce ventricular septal defects mostly likely via direct damage to the heart as a result of their ability to damage cardiomyocytes. The critical period for cardiac damage by these agents is determined to be earlier than GD 12. This would further suggest that flumioxazin might not produce cardiac ventricular defects *via* direct damage to embryonic heart tissue but rather through an indirect mechanism.

Published paper examining the effects of irradiation on rat embryonic development

On the 9th day of gestation, female rats were anaesthetised and the ventral abdominal wall opened by midline incision. One uterine horn was brought to the surface of the incision and lead plates were then arranged around and underneath the implantation sites ensuring that all remaining embryos and maternal tissues were shielded. After exposure of the unshielded embryos, they were promptly returned to their original position in the abdominal cavity. The second uterine horn was then brought to the surface, underwent similar manipulation but were not irradiated. Doses of 25, 50, 100, 200 and 400r (in air) were given at a single exposure to the unshielded embryos. Duration of exposure never exceeded 2 minutes. After post-irradiation intervals of 1 to 12 days the mothers were killed and both uterine horns inspected *in situ* for resorbed implantation sites and then dissected and examined microscopically.

Prenatal mortality was highest among irradiated animals than their non-irradiated siblings after exposure to all doses employed except 25r and it increased proportionately with higher dosage at all levels above 50r, with virtually all embryos receiving 200r dying within 4 day of treatment and the majority of embryos receiving 400r dying within 24h. Whilst multiple developmental effects were observed, discussion is focused on the cardiac effects; the paper however does not clearly state which treatment group (100r or 200r) the effects were seen in.

Cardiac malformations were noted in 17 instances. In 8 cases the abnormalities consisted of defective development in one or more of the 3 major cardiac septa, namely the interatrial, the interventricular or the AV septum.

Although not morphologically indicated on GD 9, the organs (in particular the heart) later specifically affected by irradiation must have been represented by certain cells that had already

been induced or predetermined to form particular structures later on in development. Thus it would appear that predetermined but undifferentiated primordia were more sensitive to the teratogenic effects of irradiation than organs that had already begun to differentiate. This is further supported by the observation that the heart was not structurally represented on GD9, yet was frequently malformed by irradiation at this time. Abnormalities involving the cardiovascular system were less frequent in term animals and appeared to be associated with, if not responsible for the high rate of prenatal mortality.

Published paper examining the cardiovascular anomalies produced by nimustine hydrochloride in rat fetuses

Pregnant rats received a single, intraperitoneal dose of nimustine hydrochloride (nitrosurea derivative anticancer agent that produces alkylation of DNA) of 10, 11 or 13 mg/kg. The drug was administered on GD 7, 8 or 9. Control rats received a single dose of saline on GD 8. Rats were sacrificed on GD 20. The uterine contents were examined, the number of dead or resorbed fetuses were noted, survivors removed and examined for external malformations with heart removed for morphological investigations.

Rates of resorption tended to be higher in accordance with increasing dosage. The highest frequency of cardiovascular anomalies was found in the groups treated on GD 8, but there was no difference in the rates induced by the three dosages of nimustine hydrochloride administered.

The most common cardiovascular anomalies observed were VSD and double outlet right ventricle. A considerable number of affected fetuses showed complex cardiac anomalies with AV mal-alignment and other AV value anomalies. These anomalies include: double inlet left ventricle, straddling AV value, atresia or stenosis of the AV value and dysplastic AV value.

Study examining the histopathological effects of flumioxazin on embryonic development (SBT-0064 and SBT-30-0043)

Flumioxazin was administered as a single oral, *via* gavage dose to group of both rats and rabbits on day 12 of gestation only. The dose administered to both species was 1000 mg/kg, based on previous developmental work where the developmental toxicity was produced by a single administration at 400 mg/kg in rats and for rabbits the NOAEL was in excess of 3000 mg/kg, in the presence of maternal toxicity.

Rats were sacrificed 6, 12, 24, 36 and 48 h and rabbits were sacrificed 6, 24 and 48 h post administration. Control values were obtained from untreated animals sacrificed at time point 0. Embryos with their placentas attached were removed from the uterus and examined for external malformations. The number of live and dead embryos were counted. Live fetuses were subjected to the following examinations: umbilical blood smears; yolk sac and amnion were opened and the umbilical cord exposed and embryonic blood sampled. Sections of the whole embryo including the thoraco-abdominal region were made and examined *via* light and electron microscopy.

Treatment related histopathological changes were restricted to rat embryos only. Microscopy demonstrated mitochondrial lesions including abnormal iron deposition, probably due to the inhibition of haem synthesis in erythroblasts derived from the yolk sac and subsequent degeneration of erythroblasts and erythrophagocytosis. Histological changes in the heart, such as thinning ventricular walls followed the erythroblastic lesion and likely reflected a compensatory reaction to the loss of embryonic blood cell population.

It is concluded that flumioxazin induced VSD by altering haematological function *via* the inhibition of haem synthesis rather than producing a directly injurious effect on the heart.

Study examining the pathogenesis of developmental effects produced by flumioxazin (SBT-0065)

Day 12 of gestation was selected as the day of administration on the basis of data showing that rats were the most sensitive to flumioxazin on day 12. Flumioxazin was administered as a single dose *via* oral gavage on GD 12 at a dose of 400 mg/kg.

Rats were sacrificed on GD 13, 14, 15, 16, 17 and 20. Uteri were removed and the numbers of implantations were counted. Live foetuses were then subjected to the following examinations: about half of the litters sampled on GD 14 to 20 were examined for interventricular foramen. The remaining half of the litters were examined for effects on haematology (RBC, Hb concentration) and blood chemistry (serum protein) parameters. Bone and cartilage of fetuses on GD 15 to 20 were examined.

On GD 14 treated embryos were observed to have enlarged heart, oedema and anaemia. These effects were also observed on GD 15 and 16. Post these dates treated litters were similar to controls. The mortality rate was relatively constant throughout this period, indicating that all deaths occurred during the earlier period. Closure of interventricular foramen began on GD 16 in control fetuses. In treated fetuses, closure was delayed and, the percentage of closure was well below the control value, with the foramen closed in 57.7% of treated fetuses by GD 20 compared to 95.2% in controls. Serum protein concentration was reduced on GD 15 and 16 in treated litter, with recovery by GD 17.

These data suggest that the enlarged heart, oedema and anaemia preceding the occurrence of fetal mortality may be instrumental in the cause of death. Similarly, the occurrence of enlarged heart preceding the failure of the interventricular closure would be related to the pathogenesis of this finding.

Rat teratology study with S-23121 (PPT-00-0023)

S-23121(a chemically related herbicide similar to flumioxazin) was administered orally *via* gavage to groups of pregnant female rats at concentrations of 0, 1, 3, 10 and 20 mg/kg/day from GD 6 to 15. Dams were culled on GD 20 and fetuses were removed by caesarean section and examined.

Maternal signs of toxicity were limited to high dose group dams, and included dark reddish material around the vagina. Necropsy data confirmed dark reddish material in vagina and uterus. These findings were considered to be related to the deaths of the litters. Decreases in body weight and body weight gain at term were limited again to high dose group animals; whilst not reaching statistical significance these decreases were due to reductions in gravid uterine weight which was considered to be attributable to the deaths of the litter and the decrease in fetal body weight.

High incidence of mortality of embryos and decrease of fetal body weights were limited to the high dose groups. There however was no treatment related effects on the number of implantations or sex ratio.

External abnormalities found were considered not to be treatment-related. The incidence of foetuses with cardiovascular abnormalities, primarily VSDs were increased in the 20 mg/kg/d group ($p < 0.01$). Test material related increase in wavy ribs (minor anomaly) was observed, this however did not reach statistical significance. Test material related decreases in ossified sacrococcygeal vertebral bodies were also observed. Whilst a reduction in the number of ossified sacrococcygeal vertebral bodies was observed, this was considered to be related to the decreased fetal body weights.

Based on the result of this study, the NOAEL for developmental toxicity was considered to be 10 mg/kg/d, based on increased incidence of cardiac VSD, growth retardation and embryo lethality. No signs of maternal toxicity were observed, therefore the maternal NOAEL for considered to be greater than 20 mg/kg/d.

Rabbit teratology study with S-23121 (PPT-01-0020)

S-23121 was administered orally *via* gavage to groups of pregnant female rabbits at concentrations of 0, 2, 4, 8 or 15 mg/kg/day from gestation day 7 to 19. Dams were culled on GD 29 and foetuses were removed by caesarean section and examined.

Maternal signs of toxicity included adverse clinical signs of toxicity, decreases in maternal body weight and body weight gains and feed consumption values during the dosage period in the 4 mg/kg dose group and higher. The 15 mg/kg dosage also caused the death of one dam.

No significant difference in pre or post implantation loss or early / late resorptions were observed. No test material related fetal changes were observed, with external / visceral and skeletal malformations / variations in test material treated animals being comparable to the concurrent control.

Based on the result of this study, the NOAEL for developmental toxicity was considered to be greater than 15 mg/kg/d (the highest dose tested). The maternal NOAEL was considered to be 2 mg/kg/d based on clinical signs, reductions in maternal body weight and body weight gains and relative and absolute food consumption.

Rat teratology study with S-23031 (SAT-11-0024)

S-23031 (a chemically similar herbicide to flumioxazin) was administered orally *via* gavage to groups of pregnant female rats at concentrations of 0, 50, 500 and 1500 mg/kg/day from gestation day 6 to 15. Dams were culled on GD 20 and fetuses were removed by caesarean section and examined.

No maternal signs of toxicity were observed, with food consumption, body weights or clinical signs of toxicity in treated animals being comparable to the concurrent control group.

No significant difference in pre or post implantation loss or early / late resorptions were observed. No test material related fetal changes were observed, with external / visceral and skeletal malformations / variations in test material treated animals being comparable to the concurrent control.

Based on the result of this study, the NOAEL for both developmental and maternal toxicity was considered to be greater than 1500 mg/kg/d (the highest dose tests) based on no signs of maternal or developmental toxicity observed.

Rabbit teratology study with S-23031 (SAT-11-0025)

S-23031 was administered orally *via* gavage to groups of pregnant female rabbits at concentrations of 0, 100, 200, 400 and 800 mg/kg/day from gestation day 7 to 19. Dams were culled on GD 29 and foetuses were removed by caesarean section and examined.

Four dams in the high dose group were found dead during the study, with a 5th animal from this group aborting (which was subsequently sacrificed). Clinical signs of toxicity recorded during the study were not indicative of a treatment related effect. Whilst not reaching statistical significance (due to high variability), body weight loss in the high dose group was notably greater than the control and was considered to be treatment related with high dose group animals not gaining weight during the dosing period. Mean group body weight gain for these animals was17% lower than the control for the period of GD 7 to 29.

No significant difference in pre or post implantation loss or early / late resorptions were observed. No test material related fetal changes were observed, with external / visceral and skeletal malformations / variations in test material treated animals being comparable to the concurrent control.

Based on the result of this study, the NOAEL for developmental toxicity was considered to be greater than 800 mg/kg/d (the highest dose tested). The maternal NOAEL was considered to be 400 mg/kg/d based on reductions in maternal body weight gains and maternal mortality.

4.12.4 PPO AND PPIX MECHANISTIC STUDIES

TABLE 33: OVERVIEW OF PPO AND PPIX MECHANISTIC STUDIES CONDUCTED ON FLUMIOXAZIN AND ITS METABOLITES

Method	Results	Remarks[1]	Reference
rat / rabbit (SD / JW) PPIX accumulation in maternal liver and embryos post single administration (up to 4 females/gp) oral: gavage rat / rabbit: 1000 mg/kg (exposure: single dose on GD 12) EPA OPP 83-3;non-GLP	PPIX accumulated in rat embryos up to 12 h post dosing, reaching 200-fold greater than the control values. In contrast PPIX levels in rabbits remained very low throughout the post dosing period. The species difference in PPIX accumulation in embryos correlates with that of the developmental toxicity produced by flumioxazin.	2 (reliable with restrictions) **key study** **purity: 94.8%**	Kawamura (1996a) SBT-0061 and Kawamura (1993c) SBT-30-0042
Rat (SD) PPIX accumulation in maternal liver and embryos post single administration (up to 5 females/gp) oral: gavage rat: 1000 mg/kg (exposure: single dose on GD 12) EPA OPP 83-3;non-GLP	PPIX accumulated in both whole embryos and maternal livers following administration of flumioxazin and S-23121. The extent of accumulation in embryos was greater than that observed in maternal livers, with the increase of PPIX in the embryos up to 290-fold greater than the control value. For S-23031, PPIX accumulation was not observed in either rat embryo or maternal liver samples.	2 (reliable with restrictions) **key study** **flumioxazin purity: 94.8%** **S-23031 purity: not stated** **S-23212 purity: not stated**	Kawamura (1996b) SBT-0062 and Kawamura (1993d) SBT-30-0042
rat / rabbit (SD / JW) PPIX accumulation in maternal liver and embryos (up to 5 females/gp)	PPIX accumulated in whole embryos of rats, peaking on GD 11 to 12. Accumulation of PPIX was not observed in maternal rat or rabbit livers or in rabbit embryos.	2 (reliable with restrictions) **key study**	Kawamura (1996c) SBT-0063

Method	Results	Remarks[1]	Reference
oral: gavage rat / rabbit: 400 / 1000 mg/kg (exposure: single dose on GD 10 - 15) EPA OPP 83-3;non-GLP		**purity: 94.8%**	and Kawamura (1993e) SBT-30-0042
Inhibition of PPO by flumioxazin in rat, human and rabbit liver	The IC_{50} values for flumioxazin after a 20 min incubation period for the inhibition of PPO activity in liver from rats, rabbits and humans were 0.00715 ± 0.0021 µM; 0.138 ± 0.0739 µM and 0.0173 ± 0.0044 µM, respectively. The relative sensitivity of the species to PPO inhibition by flumioxazin was rat > human > rabbit.	2 (reliable with restrictions) **key study** **purity: 94.8%**	Green & Dabbs (1996) SBT-0060
SB herbicides on PPO activity in rat and rabbit liver mitochondria	All three SB series herbicides inhibited mammalian PPO activity. The IC50 values for S-53482, S-23121 & S-23031 were respectively 23, 36 and 2230 nM for rats and 300, 690 and 12500nM for rabbits. The relative sensitivity of the species to PPO inhibition by SB series herbicides was rat > rabbit.	2 (reliable with restrictions) **key study** **S-53482 purity: 94.8%** **S-23121 purity: 94.7%** **S-23031 purity: 94.7%**	Noda (1995) SBT-0058
PPO activity in rat and rabbit tissue	Adult liver and embryo mitochondria showed similar sensitivity to PPO inhibition by the test compounds, S-53482, S-23121 and S-23031,with the rabbit enzyme results showing less sensitivity to inhibition by the test compounds than the rat enzyme. The relative potency for inhibition was flumioxazin (S-53482) > S-23121 > S-23031	2 (reliable with restrictions) **key study** **S-53482 purity: 94.8%** **S-23121 purity: 94.7%** **S-23031 purity: 94.7%**	Green & Dabbs (1993) SBT-31-0045

1. Studies evaluated according the criteria set out by Klimisch *et al* (1997)

Study examining the effects of flumioxazin on PPIX accumulation in rat and rabbit embryos (SBT-0061 and SBT-30-0042)

Day 12 of gestation was selected as the day of administration for both species on the basis of data showing that rats were the most sensitive to flumioxazin on day 12 and that rabbit embryos on day 12 were similar to rat embryos in respect to the developmental stage.

Flumioxazin was administered as a single oral, *via* gavage dose to group of both rats and rabbits on day 12 of gestation only. The dose administered to both species was 1000 mg/kg, based on previous developmental work where the NOAEL for rats was 30 mg/kg and for rabbits the NOAEL was in excess of 3000 mg/kg, in the presence of maternal toxicity.

Rats were sacrificed 2, 6, 12, 18 and 24 h and rabbits were sacrificed 2, 6, 12, 24 and 48 h post administration. Control values were obtained from untreated animals sacrificed at time point 0. Where possible, 3 embryos/litter were pooled and PPIX was extracted and analysed by HPLC with a fluorescence spectrophotometer. A sample of maternal liver was dissected out and PPIX extracted and measured.

Both rat and rabbit embryos in the developmental stage grew rapidly as shown by a marked increase in wet weight. There was a remarkable species difference in PPIX accumulation in embryos. PPIX accumulation was obvious in rat embryos within 2 h of post dosing of flumioxazin. The concentration of PPIX in rat embryos continued to increase over time and reached a peak 12 h post treatment. Thereafter the concentration of PPIX decreased rapidly.

In contrast to the rat, PPIX level in rabbit embryos were low, remaining at a fairly constant level in rabbit embryos throughout treatment.

TABLE 34: SELECTED PPIX CONCENTRATION IN RAT AND RABBIT EMBRYOS AND RAT AND MATERNAL LIVER SAMPLES

Parameter	Time after administration (h)						
	0	2	6	12	18	24	48
RAT							
PPIX/embryo (ng ± sd)	4.30 ± 1.01	80.91 ± 11.06	373.15 ± 76.10	842.56 ± 71.74	312.97 ± 174.09	181.63 ± 125.60	-
PPIX conc. (µg/g tissue ± sd)	0.147 ± 0.038	2.831 ± 0.236	10.806 ± 0.906	19.080 ± 1.583	5.061 ± 2.321	2.739 ±1.852	-
PPIX/maternal liver sample (ng ± sd)	38.70 ± 6.81	401.70 ± 43.04	528.83 ± 74.76	470.93 ± 64.82	117.85 ± 10.68	140.93 ± 83.24	-
PPIX conc. (µg/g tissue ± sd)	0.111 ± 0.013	1.078 ± 0.168	1.383 ± 0.198	1.259 ± 0.179	0.359 ± 0.064	0.358 ± 0.211	-
RABBIT							
PPIX/embryo (ng ± sd)	Not detected	5.47[a]	5.93 ± 0.00[b]	8.26 ± 2.95	-	7.87	Not detected
PPIX conc. (µg/g tissue ± sd)	Not detected	0.092[a]	0.104 ± 0.021[b]	0.110 ± 0.031	-	0.061[a]	Not detected
PPIX/maternal liver sample (ng ± sd)	21.07 ± 1.89	50.40 ± 10.61	39.70 ± 14.66	80.80 ± 7.35	-	57.65 ± 44.19	28.73 ± 4.14
PPIX conc. (µg/g tissue ± sd)	0.054 ± 0.004	0.183 ± 0.092	0.114 ± 0.029	0.223 ± 0.022	-	0.160 ± 0.102	0.079 ± 0.016

a value of 1 sample. PPIX not detected in other pooled sample
b mean value of 2 samples
sd standard deviation

A similar pattern was observed in PPIX accumulation in maternal liver of rats, with levels of PPIX peaking at 6 h post treatment, however the levels of PPIX in the liver peaked at ~14 fold greater than the control, these levels were ~14 fold less than the peak accumulation observed in the rat embryo. There was no accumulation of PPIX in the maternal livers of rabbits.

In conclusion, the species difference in PPIX accumulation in embryos correlates with that of the developmental toxicity produced by flumioxazin.

Study examining flumioxazin and other N-phenylimide herbicides on PPIX accumulation in rat embryos (SBT-0062 and SBT-30-0042)

Day 12 of gestation was selected as the day of administration on the basis of data showing that rats were the most sensitive to flumioxazin on day 12. Flumioxazin, and 2 other N-phenylimide herbicides, S-23121 and S-23031 were administered orally *via* gavage on GD 12 at a dose of 1000 mg/kg.

Rats were sacrificed 14 h post dosing and 3 embryos/litter were removed from the uterus and pooled by litter. PPIX was extracted from embryo pools and PPIX was extracted and analysed by HPLC with a fluorescence spectrophotometer. A sample of maternal liver was dissected out and PPIX extracted and measured.

No treatment related accumulation of PPIX was observed in rat embryos exposed to 1000 mg/kg S-23031, a chemical which has shown no developmental toxicity in rats. The PPIX content/g of tissue in rat embryos treated with S-23031 was 0.118 μg/g, which was similar to the control value (0.095 μg/g). In contrast, treatment with 1000 mg/kg of either S-23121 or flumioxazin, which have both produced the same pattern of developmental toxicity in rats, resulted in large increases in PPIX accumulation in treated embryos. PPIX concentration in embryos treated with S-23121 and flumioxazin was 25.85 and 27.62 μg/g, respectively. These values were ~270 and ~290 times greater than the control value, respectively.

Maternal liver PPIX levels for both S-23121 and flumioxazin were comparable and were in the region of 3.6 and 2.9-fold greater than the control, respectively. For S-23031 the extent of PPIX accumulation in the liver was comparable to the control value.

TABLE 35: PPIX CONCENTRATION IN RAT EMBRYOS AND MATERNAL LIVER SAMPLES

Parameter	Control (0.5% MC[1])	S-23031 (1000 mg/kg)	S-23121 (1000 mg/kg)	S-53482 (1000 mg/kg)
PPIX/embryo (ng ± sd)	5.60 ± 1.38	6.33 ± 1.63	1656.76 ± 307.52	1667.37 ± 113.74
PPIX conc (μg/g tissue ± sd)	0.095 ± 0.030	0.118 ± 0.030	25.850 ± 2.735	27.619 ± 3.894
PPIX/maternal liver sample (ng ± sd)	74.75 ± 23.76	99.68 ± 22.71	212.20 ± 99.31	169.60 ± 42.83
PPIX conc. (μg/g tissue ± sd)	0.236 ± 0.067	0.379 ± 0.068	0.857 ± 0.418	0.679 ± 0.109

1. MC – methyl cellulose
sd standard deviation

In conclusion, treatment of pregnant rats with S-23121 or flumioxazin, two structurally related chemicals which produce developmental effects in rats, resulted in the accumulation of PPIX in both the whole embryos and maternal livers. The extent of accumulation in embryos was greater than that observed in maternal livers, with the increase of PPIX in the embryos up to 290-fold greater than the control value. For S-23031, another structurally related chemical, which does not produce developmental toxicity in rats, PPIX accumulation was not observed in either rat embryo or maternal liver samples.

Study examining the effects of flumioxazin on PPIX accumulation in rat and rabbit embryos (SBT-0063 and SBT-30-0042)

Flumioxazin was administered as a single oral dose, *via* gavage to groups of both rats and rabbits to investigate the correlation of PPIX accumulation with the critical period of embryonic sensitivity in the rat. The dose administered to rats and rabbits was 400 and 1000 mg/kg, respectively from gestation day 10 to 15.

Animals were sacrificed 14 h post final administration. Control values were obtained from untreated animals sacrificed at time point 0. Where possible, 3 embryos/litter were pooled and PPIX was extracted and analysed by HPLC with a fluorescence spectrophotometer. A sample of maternal liver was dissected out and PPIX extracted and measured.

PPIX concentrations in treated rat embryos were more than 10 times higher than controls on treatment days 10 through to 15 of gestations. PPIX accumulation peaked on gestational days 11 to 12. The critical period for induction of PPIX accumulation determined in the current study corresponds well with the critical period for induction of developmental toxicity in the rat embryo. This would imply that the PPO inhibition and the resulting PPIX accumulation may be functionally related to the developmental toxicity produced by flumioxazin

TABLE 36: PPIX CONCENTRATION IN RAT EMBRYOS AND MATERNAL LIVER SAMPLES

Parameter		Gestation day of administration					
		10	11	12	13	14	15
RAT							
S-53482 (400 mg/kg)	PPIX conc. /embryo (µg/g tissue ± sd)	3.335 ± 1.204	10.214 ± 7.007	8.701 ± 5.837	0.610 ± 0.249	0.684 ± 0.497	1.853 ± 1.229
	PPIX conc. /maternal liver (µg/g tissue ± sd)	0.245 ± 0.072	0.364 ± 0.246	0.359 ± 0.090	0.220 ± 0.048	0.287 ± 0.051	0.275 ± 0.034
RABBIT							
S-53482 (1000 mg/kg)	PPIX conc. /embryo (µg/g tissue ± sd)	Not detected	0.060a	Not detected	0.047a	0.045a	0.077 ± 0.019
	PPIX conc. /maternal liver (µg/g tissue ± sd)	0.080 ± 0.028	0.256 ± 0.166	0.175a ± 0.089	0.137 ± 0.040	0.121b ± 0.027	0.109a ± 0.057

a mean value of three pools. PPIX was not detected in other pool
b mean value of two pools. PPIX was not detected in other pool
sd standard deviation

PPIX accumulation was not observed at any developmental stage in the rabbit. The rabbit has been shown in previous studies to be resistant to any developmental toxicity from flumioxazin. The lack of accumulation of PPIX in the rabbit further supports the relationship between PPO inhibition, PPIX accumulation and developmental toxicity of flumioxazin in the rat.

Accumulation of PPIX was not observed in maternal rat or rabbit liver at the doses used in this study.

Study examining the inhibition of PPO by flumioxazin *in vitro* (SBT-0060)

The objective of the study was to determine the relative extent of inhibition of PPO activity by flumioxazin in adult liver mitochondria prepared from rat, rabbit and human tissues.

PPO was measured in viable and metabolically active mitochondria prepared from rat, rabbit and adult female human livers. For human liver mitochondria, the time course of reaction, the mitochondrial protein concentration, and the substrate concentration effects were determined. The Michaelis constant K_m and V_{max} of the reaction was determined per human liver, K_m values were 0.328 ± 0.301 µM and the V_{max} values were 111 ± 88.9 pmol/min-1/mg protein. The inhibition of PPO activity was measured at a range of flumioxazin test concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}M.

The IC_{50} values for flumioxazin after a 20 min incubation period for the inhibition of PPO activity in liver from rats, rabbits and humans were 0.00715 ± 0.0021 µM; 0.138 ± 0.0739 µM

and 0.0173 ± 0.0044 µM, respectively. The relative sensitivity of the species to PPO inhibition by flumioxazin was rat > human > rabbit.

Study examining SB herbicides on PPO activity in mitochondria derived from rat and rabbit liver (SBT-0058)

The objective of the study was to determine the relative extent of inhibition of protoporphyrinogen oxidase activity by three SB series herbicides (S-53482 (flumioxazin), S-23121 & S-23031) in adult liver mitochondria prepared from rat and rabbit tissues.

PPO was measured in viable and metabolically active mitochondria prepared from rat and rabbit female livers. The inhibition of PPO activity was measured at a range of test concentrations of 10^{-4} to10^{-10}M.

All three SB series herbicides inhibited mammalian PPO activity. The IC_{50} values for S-53482, S-23121 & S-23031 were respectively 23, 36 and 2230 nM for rats and 300, 690 and 12500 nM for rabbits. The relative sensitivity of the species to PPO inhibition by SB series herbicides was rat > rabbit.

Study examining SB herbicides on PPO activity in rat and rabbit tissue (SBT-31-0045)

The objective of the study was to determine the relative extent of inhibition of interspecies and tissue differences in protoporphyrinogen oxidase activity and its inhibition by three test materials, S-53482, S-23121 and S-23031, in adult liver and embryo mitochondria prepared from rat and rabbit tissues.

PPO was measured in viable and metabolically active mitochondria prepared from rat and rabbit livers and embryos on gestational days of 12 and 15. The time-course PPO activity reached steady-state after 20 min in the rat. In contrast, rabbit liver PPO activity reached steady-state at 40 min. The time course of PPO activity was found to be linear in embryo tissues of both gestational ages and both species for at least 40 min. The Michaelis constant K_m and V_{max} of the reaction was determined at a single time point with six different concentrations of protoporphyrinogen ((5, 10, 25, 50, 75, 100 pmol). The K_m values ranged from 1 – 5 µM, except for rat and rabbit 15-day embryos where the K_m values were higher (8.2 ± 1.0 and 12.3 ± 0.46 µM for the rat and rabbit, respectively).

The inhibition of PPO activity was measured against the three test materials at final test concentrations of: S-53482 and S-23121: 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, $10^{-9}$and 10^{-10}M. For S-23031: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, $10^{-8}$and 10^{-9}M.

Adult liver and embryo mitochondria showed similar sensitivity to PPO inhibition by the test compounds, suggesting that adult liver mitochondria could serve as a source of PPO in future experiments and eliminating the requirement for embryonic tissue. The rabbit was less sensitive to inhibition by the test compounds than the rat. The relative potency for inhibition was flumioxazin (S-53482) > S-23121 > S-23031.

4.12.5 SUMMARY AND DISCUSSION OF MECHANISTIC WORK

Summaries and evaluations of the mechanistic research on flumioxazin are presented in Annexes 1 and 2 to this report. Annex 1 (SBT-0122) is focused primarily on discussing the human relevance of the mode of action for teratogenic effects in the rat based on new data generated since the publication of flumioxazin in the 28th ATP of the DSD. Annex 2 (SBT-0067) presents

the evidence for the proposed mode of action in the rat based on studies conducted prior to the publication in the 28th ATP of the DSD.

An overall summary of the mode of action and relevance to humans for the teratogenic effects in the rat is presented below.

Flumioxazin caused embryolethality, teratogenicity (mainly VSD and wavy ribs), and growth retardation in rats at 30 mg/kg without maternal toxicity but not in rabbits at the maternal toxicity level of 3000 mg/kg.

There is convincing evidence for a single mode of action causing the developmental toxicities in the rat. The sequence of key biological events in the proposed mode of action has been elucidated. Inhibition of PPO interferes with normal haem synthesis, which causes loss of fetal blood cells due to the targeted destruction of maturing erythroblasts in the yolk sac. The maturation of erythroblasts in the yolk sac is a synchronised event, therefore catastrophic loss of this cell population leads to fetal anaemia, embryolethality and development of malformations (VSD and wavy ribs). The critical period for sensitivity to the developmental effects of flumioxazin is day 12 of gestation and this correlates with the peak period of PPIX accumulation in the rat fetus resulting from the inhibition of PPO. The VSD caused by flumioxazin appears to result from inhibition of haem biosynthesis rather than from direct injury to embryonic heart tissue. Enlargement of the heart is a compensatory reaction to the fetal anaemia resulting from increased heart stroke volume observed in surviving fetuses. Enlargement of the heart precedes interventricular foramen closure. Therefore, the VSD is due to failure of heart closure resulting from mechanical distortion of the heart and/or abnormal blood flow rather than from direct toxic effects of flumioxazin on cardiac muscle tissue. In addition, decreased serum protein is observed in the fetus, presumably due to reduced production in the liver in response to hypoxia. The resulting osmotic imbalance causes oedema. Reduction of fetal serum protein leads to incomplete/delayed ossification of the ribs and the wavy ribs seen at term. The sensitivity to inhibition of PPO extracted from adult female livers is comparable with that of the fetus and there does not appear that there is a significant difference in sensitivity to the development of anaemia between adult and fetal rats.

An evaluation of other potential modes of action (Annex 1) revealed no compelling evidence for any other mode of action for the developmental toxicity of flumioxazin in the rat.

Studies in the rabbit (where clear evidence of maternal toxicity was observed) revealed no significant inhibition of PPO by flumioxazin and there was no evidence of fetal anaemia, accumulation of PPIX or developmental toxicity in the rabbit fetus, even though there was evidence of placental transfer of flumioxazin and its metabolites.

Rats are particularly sensitive to the effects of PPO inhibition induced by flumioxazin in erythroblasts. This leads to anaemia that is a critical precursor of the developmental toxicity resulting from flumioxazin exposure. The systemic-dose-response for this key event has proved to be very steep: half-dose has been without any effect.

In contrast, humans are unlikely to develop anaemia from PPO inhibition. This conclusion is based on:

1. clinical findings that PPO deficient patients with Variegate Porphyria show no signs of anaemia,
2. experimental evidence that flumioxazin and its metabolites do not reduce heme production in K562 cells, which are derived from human erythroleukemia, and

3. that humans are less sensitive to PPO inhibition than rats.

Pharmacokinetic modelling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at an exposure equivalent to a maternal dose exceeding 1000 mg/kg/day, thus demonstrating the large species difference in sensitivity. As a result of the decrease in absorption rate with oral dose, the systemic daily dose cannot exceed value of approximately 100 mg/kg bw.

In addition, a recent medical surveillance report conducted on manufacturing plant personnel (Nishioka, 2011 SBT-0116) revealed no evidence of haematotoxicity, or other adverse health effects in workers (n=15) who have been involved in the manufacture of flumioxazin for the last decade. This is considered to demonstrate not only effective uses of personal protective equipment, but also the intrinsic low toxicity of flumioxazin.

Overall, it is concluded that the rat is an inappropriate model for assessing the developmental toxicity of flumioxazin in humans because, unlike humans, they are highly sensitive to PPO inhibition, resulting in fetal anaemia and consequent developmental toxicity. There is considered to be no plausible scenario whereby humans would be at risk of developmental toxicity given the species differences in susceptibility to flumioxazin and potential for anaemia.

4.12.6 ASSESSMENT AGAINST CLP CRITERIA FOR CLASSIFICATION OF A SUBSTANCE FOR REPRODUCTIVE TOXICITY

The implications of the developmental findings in rats exposed to flumioxazin for hazard classification are evaluated using the criteria of Regulation 1272/2008 and the ECHA Guidance on the Application of the CLP Criteria in Regulation (EC) No. 1272/2008 (ECHA, 2011).

Classification of a substance as a reproductive toxicant to humans (Category 1A – known, Category 1B – presumed, or Category 2 – suspected) is based on a weight of evidence approach and expert judgment. Reproductive toxicity is subdivided under 2 main headings:

•*Adverse effects on sexual function and fertility*

•*Adverse effects on development of the offspring*

Consideration is also given to effects on lactation.

In the case of flumioxazin the relevant effects concern development of the offspring.

The guidance considers whether maternal toxicity may influence classification but the developmental toxicity associated with flumioxazin was observed in the absence of gross signs of maternal toxicity even though there was likely to be an underlying anaemia in the dams. Given the specificity of the cardiac malformations it is considered unlikely that secondary consequences of maternal anaemia on the developing fetus would have been responsible. Although fetal growth retardation, reduced ossification and wavy ribs may be associated with overt maternal toxicity this does not seem to be the case with flumioxazin. Therefore, maternal toxicity is considered not to be a confounding factor in determining the classification of flumioxazin.

The guidance also considers whether there is mechanistic information needs to be evaluated in deciding the classification for reproductive toxicity. The extensive mechanistic research on flumioxazin has clearly demonstrated that:

1. there is convincing evidence for a single mode of action causing the developmental toxicities in the rat, and

2. humans are unlikely to develop anaemia from PPO inhibition and thus would not be susceptible to the mode of action causing developmental toxicity in the rat.

The criteria for Categories 1B and 2 are as follows:

Category 1B

"Presumed human reproductive toxicant

The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects , or if occurring with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. **However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate**."

Category 2

"Suspected human reproductive toxicant

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 ot to be a secondary non-specific consequence of other toxic effects."

It is concluded that the mechanism of developmental toxicity via severe anaemia observed in rats is not relevant for human health hazard prediction. On this basis flumioxazin could be assigned Category 2 taking account of the statement in bold for category 1B. However, according to Regulation 1272/2008 (3.7.2.5.5): ". . . if it can be conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expressed in humans then a substance which produces an adverse effect on reproduction in experimental animals shall not be classified."

The weight of evidence presented in the CLH report demonstrates conclusively that flumioxazin would not present hazard to humans based on the marked differences in species sensitivity. Therefore, it should not be classified for developmental toxicity.

The weight of evidence approach followed the principles of an established human relevance framework (HRF) for non-cancer endpoints prepared by the International Programme on Chemical Safety (Boobis et al, 2008). The publication describes a structured weight of evidence approach to assessing the human relevance of a postulated MOA in animals. Whilst the HRF is primarily aimed at chemical risk assessment it is equally applicable to hazard classification where the human relevance of a MOA in animals requires evaluation. The analogous cancer HRF is cited in the ECHA guidance (2011) for the classification of carcinogens (page 299 § 3.6.2.3.).

The non cancer HRF requires 3 fundamental questions to be addressed in order to reach a conclusion on the human relevance of toxicological effects observed in animals:

1. Is the weight of evidence sufficient to establish a mode of action (MOA) in animals?

2. Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?

3. Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

In the case of flumioxazin the answer to question 1 is yes. The weight of evidence is sufficient to establish the MOA for developmental toxicity in the rat. The answer to question 2 is no, it cannot be concluded that there is a fundamental qualitative species difference. The answer to question 3 is yes based on the evidence for a marked difference in sensitivity between rats and humans attributed to quantitative dynamic and kinetic differences. Although Boobis et al (2008) note that dismissing human relevance based on quantitative differences is likely to be infrequent, they go on to mention that this is achievable where human exposure could not possibly be envisaged to reach the levels that would produce the toxicological effect. The pharmacokinetic modelling presented in the CLH report demonstrates that there is no plausible scenario whereby human exposure to flumioxazin could cause the developmental toxicity ascribed to the MOA in the rat.

Another publication by Lavelle et al (2012) endorses the HRF prepared by the IPCS. Lavelle et al present an algorithm for categorising the relevance of animal data for use in human risk assessment (Figure 1 in the paper). It incorporates the principles of the HRF and the relevant aspects of the algorithm are shown below:

Is the MOA established in animals?

- No. Assume relevant to man
- **Yes, see below**

Are the key events plausible in man?

- No. Not relevant to man
- **Yes, see below**

Taking into account kinetic and dynamic factors is the animal MOA plausible in man?

- **No. Not relevant to man**
- Yes, directly. Relevant to man
- Yes, with a sensitivity difference. Relevant to man
- Maybe. Assume relevant to man

The elements in bold are applicable to flumioxazin and support the conclusions presented above.

4.12.7 CONCLUSIONS ON CLASSIFICATION

There is a convincing weight of evidence to conclude that flumioxazin would not present a reproductive hazard to humans and should not be classified for reproductive toxicity based on

the criteria for classification in Regulation EC 1272/2008. Therefore, removal of the current reproductive toxicity classification (Repr. 1B H360D) is warranted.

RAC evaluation of reproductive toxicity

Summary of the Dossier submitter's proposal

The substance has a harmonised classification for reproductive toxicity in category 1B based on teratology studies (consistent with guideline EPA OPP 83-3) in SD rats (oral and dermal) and it is not contested in the dossier, that these results are severe enough to warrant classification. Since the time the current classification was decided, new data have been generated leading the DS to suggest the removal of the classification as toxic to reproduction. The new data consisted of a negative teratology study in NZW rabbits (guideline EPA OPP 83-3) and a large set of mechanistic data. The DS argued that the effects seen in rats are not relevant for humans.

The principal argument does not question that flumioxazin causes significant developmental toxicity/teratogenicity in rats but rather that there is a clear species difference with respect to susceptibility to the specific mechanism, with rats more sensitive than humans which are in turn more sensitive than rabbits.

The database contains the following;

1. **The original guideline data** set consisting of a 2-generation study in rats, oral and dermal developmental toxicity studies in rats and a developmental toxicity study in rabbits.

2. **The original data set of mechanistic studies** with flumioxazin:
   -Haematotoxicity of flumioxazin
   -Placental transfer
   -Critical period of embryonic sensitivity
   -Histopathological study of early stages of development in rat and rabbits foetuses following exposure to flumioxazin
   -Pathogenesis of developmental effects of flumioxazin
   -Studies of PPO inhibition/PPIX accumulation in embryos
   -Species differences in PPIX accumulation
   -Critical period for PPIX accumulation in rat and rabbit embryos

3. **Recent mechanistic studies**
   -Pharmacokinetics rat/rabbit
   -Chronological changes of morphology and population of circulating erythroblasts in rat embryos during yolk sac haematopoiesis.
   -Inhibition of PPO activity by flumioxazin and its major metabolites, 3-OH S-53482, 4-OH S-53482 and APF in rat liver mitochondria.
   - Species differences in PPIX accumulation induced by flumioxazin in cryopreserved hepatocytes among rat, rabbit, monkey and human.
   - Effects of flumioxazin and metabolites of flumioxazin on haem synthesis pathway and cell proliferation in K562 cells.
   -Physiologically based pharmacokinetic (PBPK) modelling of flumioxazin in rats and humans and *in silico*.

Summary of guideline Developmental toxicity studies.

Flumioxazin induced embryolethality and teratogenicity in the rat following dosing *via* both the oral (at 30 mg/kg bw/day) and dermal (at 300 mg/kg bw/day) routes. Abnormalities mainly consisted of cardiac ventral septal defect (VSD). In addition, there was an increase in the

incidence of wavy ribs and reduced ossification of sacrococcygeal vertebral bodies Furthermore, foetal growth retardation was also observed in both studies. This observation was supported by the occurrence of reduced litter size (embryofoetal lethality) and reduced pup weight seen in the 2-generation study. These effects were observed in the rat at relatively low levels and in the absence of maternal toxicity. In contrast, flumioxazin showed no evidence of developmental toxicity in the rabbit even in the presence of maternal toxicity. The maximum dose administered in the rabbit study was 100-fold greater (3000 mg/kg/d) than the maximum dose administered in the rat oral developmental study.
In addition, developmental toxicity studies were conducted with two closely related structural analogues of flumioxazin. S-23031 was shown to be negative for developmental toxicity in both the rat and rabbit. S-23121 was shown to cause increased incidence of cardiac VSD, growth retardation and embryo lethality in the absence of signs of maternal toxicity in the rat, and had no adverse effect on development up to doses causing maternal toxicity in the rabbit.

Proposed mechanism of action.

Flumioxazin is a herbicide which disrupts photosynthesis probably by inhibition of PPO and auto-oxidation of protoporphyrinogen IX (PPPIX) to protoporphyrin IX (PPIX). Porphyrin biosynthesis is common to plants and animals, as part of chlorophyll and as part of the penultimate enzyme in haem synthesis, respectively. The mode of action of flumioxazin and N-phenylimide herbicides is presented in Figure 1.

The mechanism by which developmental toxicity is produced by flumioxazin is presented in Figure 2 and is postulated as follows: flumioxazin inhibits a key enzyme, (PPO) in rats, interfering with normal haem synthesis in the mitochondria. Inhibition of PPO leads to an accumulation of its substrate, PPPIX in the mitochondrion. The accumulated PPPIX leaves the mitochondrion and undergoes non-enzymatic oxidation to PPIX in the plasma. The resulting PPIX is out of reach of the final enzyme in haem synthesis (ferrochelatase) and cannot be transferred to haem, resulting in anaemia. The foetal anaemia leads to hypoxia in foetal tissues followed by suppressed liver function and a decrease in protein synthesis. This decreased protein synthesis would result in wavy ribs and changes in osmotic forces are thought to be responsible for the oedema observed in the foetus. Concurrently, the foetus may compensate for the anaemia by pumping a greater volume of blood leading to the observed enlargement of the heart just prior to closure of the interventricular foramen, thus resulting in the delayed closure of the foramen and VSD. Thus, the VSD observed in teratology studies is considered to be produced by mechanical distortion of the heart. The two other signs of developmental toxicity reported (growth retardation and foetal death) are also considered related to the hypoxia produced by the anaemic condition in the foetus.

Figure 1 Mode of action of N-phenylimide herbicides

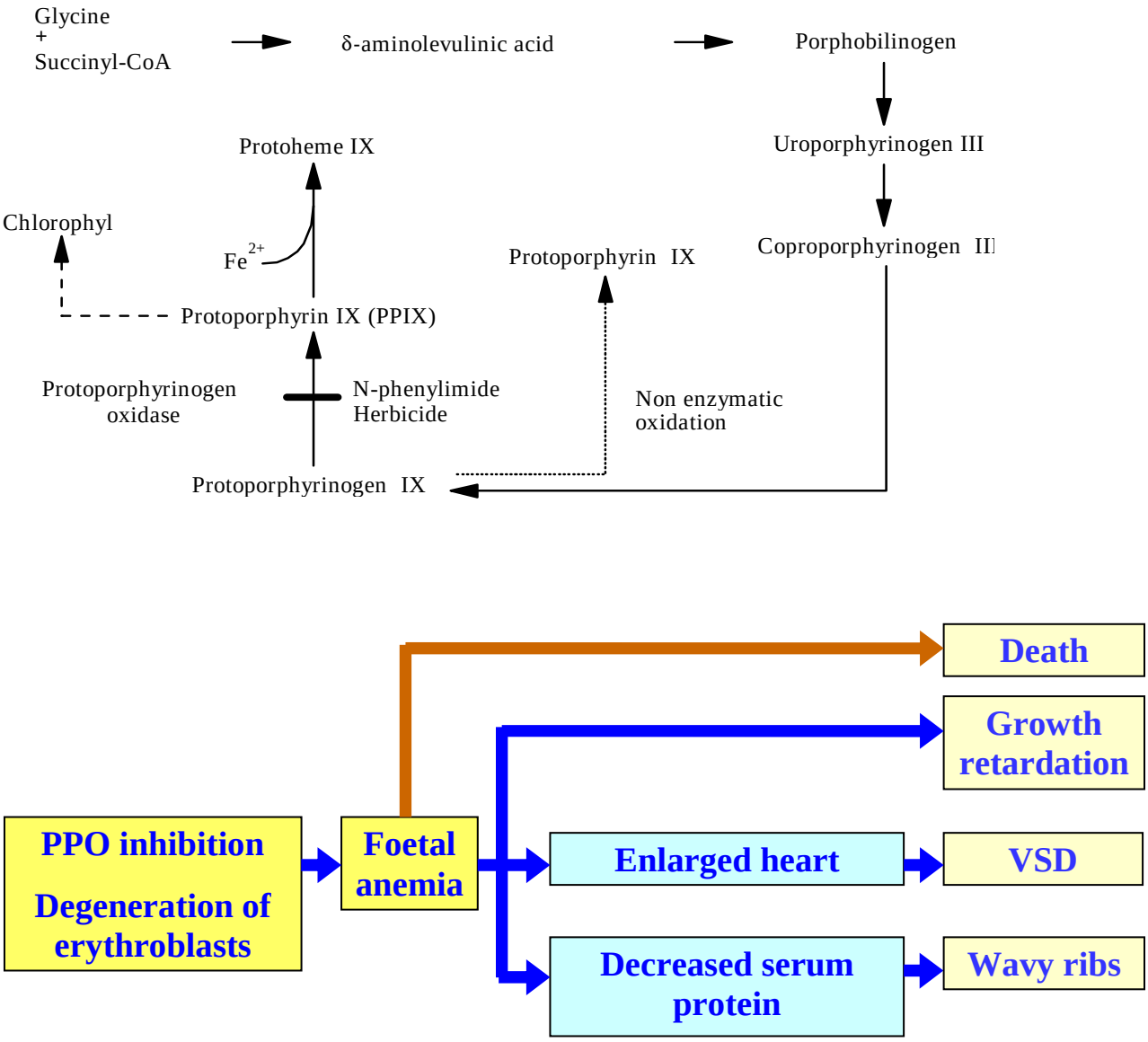


Figure 2. Proposed mechanism of developmental toxicity induced by flumioxazin

The mechanism described in Figure 2 is based on a series of studies designed to elucidate the basis for the observed difference in developmental toxicity produced by flumioxazin in rats but not in rabbits. The studies described below were evaluated during the previous review of flumioxazin for Annex 1 inclusion (91/414/EC) and were summarized in the DAR.

The original data set of mechanistic studies.

Method	Results	Reference
Haematotoxicity and placental transfer studies		
rat (SD) study examining the mechanism of haematotoxicity (up to 30 animals/sex/gp) oral: feed	Flumioxazin induced anaemia in rats can be classified as sideroblastic anaemia resulting primarily from the defective haem pathway during the process of haemoglobin biosynthesis considering the increase in porphyrins and	Yoshida (1996) SBT-0059

study 1: 0, 3000, 10000 ppm study 2: 0, 3000 ppm (exposure: study 1: 37 days; study 2: 15 days) No guideline available; non-GLP. **Flumioxazin 94.8% pure**	siderocytes. The increased blood porphyrin level suggested that the S-53482 induces porphyria in rats.	
rat / mouse (SD / ICR) study examining placental transfer of flumioxazin (24 animals/gp) oral: gavage 30 mg/kg (exposure:GD12) EPA OPP 85-1, non-GLP **[phenyl-U ^{14}C]flumioxazin purity: >99%**	In mice significantly higher transfer of radioactivity to blood cells was observed compared with rats. Elimination of radioactivity from female reproductive tissue of both species was slower than that from blood (blood cell & plasma), with only a small amount of radioactivity being transferred to the foetus.	Isobe (1992) SBM-20-0015
rat / rabbit (SD / JW) study examining placental transfer of flumioxazin oral: gavage 30 mg/kg (exposure: rats GD 12 / mice GD10) EPA OPP 85-1, non-GLP **[phenyl-U ^{14}C]-flumioxazin purity: >99%**	In rats significantly higher transfer of radioactivity to blood cells was observed compared with rabbits. Elimination of radioactivity from female reproductive tissue of both species reached maxima 2-4 hrs after administration and decreased rapidly thereafter, with only a small amount of radioactivity being transferred to the fetus.	Isobe (1993) SBM-30-0032
Critical window/histopathogenesis of critical effect		
Critical window study: rat (SD) study examining the critical period for developmental toxicity (5 females/gp) oral: gavage 400 mg/kg (exposure: single dose on GD 11, 12, 13, 14 or 15) EPA OPP 83-1, non-GLP **Flumioxazin 94.8%**	The data confirmed that the most sensitive developmental stage common to VSD, embryonic mortality and reduced fetal body weight was on GD 12. Since all 3 endpoints peaked in incidence on GD 12, it is suggested that the mechanism involved in all 3 parameters is common to teratogenicity, embryolethality and growth retardation.	Kawamura (1993a) SBT-30-0044

Histopathology rat / rabbit (SD / JW) study examining the histopathological effects of flumioxazin on embryonic development oral: gavage 0, 1000 mg/kg (single exposure GD 12) EPA OPP 83-3; non-GLP **Flumioxazin 94.8%**	Histopathology in rat embryos only: -mitochondrial lesions including abnormal iron deposition possibly related to inhibition of haem synthesis in yolk sac erythrocytes with subsequent degeneration. -thinning of the ventricular wall following erythroblastic lesion may reflect reaction to loss of embryonic blood cell population. <u>Conclusion</u> Flumioxazin did not induce VSD due to a direct injurious effect on embryonic heart tissue. The effects were likely attributed to an indirect mechanism, where flumioxazin inhibits PPO in rat embryos only, thereby interfering with normal haem biosynthesis resulting in embryonic anaemia. The embryo compensates for the anaemic hypoxia by increasing heart stroke volume, leading to hypertrophy of the heart. VSD defects result from mechanical distortion of the heart.	Kawamura & Yoshioka (1997) SBT-0064 and Kawamura (1993b) SBT-30-0043
Histopathology rat (SD) study examining the pathogenesis of developmental effects produced by flumioxazin oral: gavage 0, 400 mg/kg (single exposure GD 12) EPA OPP 83-3; non-GLP **Flumioxazin 94.8%**	Data from this study suggest that the enlarged heart, oedema and anaemia preceding the occurrence of fetal mortality may be instrumental in the cause of death. Similarly, the occurrence of enlarged heart preceding the failure of the interventricular closure would be related to the pathogenesis of this finding.	Kawamura (1997) SBT-0065
PPO and PPIX mechanistic studies		
rat / rabbit (SD / JW) PPIX accumulation in maternal liver and embryos post single administration (up to 4 females/gp) oral: gavage rat / rabbit: 1000 mg/kg (exposure: single dose on GD 12) EPA OPP 83-3;non-GLP **Flumioxazin purity: 94.8%**	PPIX accumulated in rat embryos up to 12 h post dosing, reaching 200-fold greater than the control values. In contrast PPIX levels in rabbits remained very low throughout the post-dosing period. The species difference in PPIX accumulation in embryos correlates with that of the developmental toxicity produced by flumioxazin.	Kawamura (1996a) SBT-0061 and Kawamura (1993c) SBT-30-0042
Rat (SD) PPIX accumulation in maternal liver and embryos post single administration (up to 5 females/gp) oral: gavage rat: 1000 mg/kg (exposure: single dose on GD 12) EPA OPP 83-3;non-GLP **Flumioxazin purity: 94.8%**	PPIX accumulated in both whole embryos and maternal livers following administration of flumioxazin and S-23121. The extent of accumulation in embryos was greater than that observed in maternal livers, with the increase in PPIX in the embryos up to 290-fold greater than the control value. For S-23031, PPIX accumulation was not observed in either rat embryo or maternal liver samples.	Kawamura (1996b) SBT-0062 and Kawamura (1993d) SBT-30-0042
rat / rabbit (SD / JW) PPIX accumulation in maternal liver and embryos (up to	PPIX accumulated in whole embryos of rats, peaking on GD 11 to 12. Accumulation of PPIX was not observed in maternal rat or rabbit livers or in	Kawamura (1996c) SBT-0063

5 females/gp) oral: gavage rat / rabbit: 400 / 1000 mg/kg (exposure: single dose on GD 10 - 15) EPA OPP 83-3;non-GLP **Flumioxazin** **purity: 94.8%**	rabbit embryos.	and Kawamura (1993e) SBT-30-0042
Inhibition of PPO by flumioxazin in rat, human and rabbit liver **Flumioxazin** **purity: 94.8%**	The IC_{50} values for flumioxazin after a 20 min incubation period for the inhibition of PPO activity in liver from rats, rabbits and humans were: Rats: 0.00715 ± 0.0021 µM; Humans: 0.0173 ± 0.0044 µM. Rabbits: 0.138 ± 0.0739 µM The relative sensitivity of the species to PPO inhibition by flumioxazin was rat > human > rabbit.	Green & Dabbs (1996) SBT-0060
Effect of SB herbicides on PPO activity in rat and rabbit liver mitochondria **-Flumioxazin (94.8%)** **- S-23031 (dev tox neg)** **-S-23121 (dev tox pos**)	All three SB series herbicides inhibited mammalian PPO activity. The IC_{50} values: Flumioxazin (rat): 23 nM S-23121 (rat): 36 nM S-23031 (rat): 2230 nM Flumioxazin (rabbit) 300 nM S-23121 (rabbit): 690 nM S-23031 (rabbit): 12500 nM The relative sensitivity of the species to PPO inhibition by SB series herbicides was rat > rabbit.	Noda (1995) SBT-0058
PPO activity in rat and rabbit tissue **-Flumioxazin (94.8%)** **- S-23031 (dev tox neg)** **-S-23121 (dev tox pos)**	Adult liver and embryo mitochondria showed similar sensitivity to PPO inhibition by the test compounds, Flumioxazin, S-23121 and S-23031. Rabbit enzyme results showing less sensitivity to inhibition by the test compounds than the rat enzyme. The relative potency for inhibition was flumioxazin > S-23121 > S-23031	Green & Dabbs (1993) SBT-31-0045

The main points identified in these studies were as follows:

1. *Critical window and pharmacokinetics:* The critical period for sensitivity to the pre-natal developmental effects of flumioxazin including foetal death, reduced foetal bodyweight and VSD following single administrations of 400 mg/kg, was day 12 of gestation. This suggested a common mechanism for the three types of developmental effects. The effects seen following a single administration were identical to the developmental effects identified following repeated exposure in the guideline developmental toxicity study at 30 mg/kg, however, the dose levels used were significantly different.

   Absorption at doses of 30, 400, and 1000 mg/kg, was 50% (actual value), 21% (logarithmic approximation), and 12% (actual value), respectively (Takaku 2012a, SBM-0089; Takaku 2012b, SBM-0092 and Takaku 2012c: SBM-0093).

   Therefore, the internal dose at 400 mg/kg was estimated to be 84 mg/kg and was in fact 5.6 times higher than that following dosing with 30 mg/kg (internal dose 15 mg/kg). In addition, calculation using the physiologically-based pharmacokinetic (PBPK)

model estimated the Cmax and AUC at 400 mg/kg to be 4 times and 6 times larger than that at30 mg/kg. The DS considered, therefore, that a single treatment at 400 mg/kg could be comparable to a repeated treatment at 30 mg/kg during the sensitive period.

Following dosing with 30 mg/kg in the comparative pharmacokinetics study, flumioxazin derived radioactivity was detected at a concentration of 0.02 µg eq./g in both rat maternal plasma and foetus (Shirai, 2009). Therefore, there was no difference in exposure to flumioxazin between maternal animals and foetuses.

Table 1. Internal dose of flumioxazin in foetus at different oral dose levels

Dose regimen	Cmax (µg/mL)	AUC (µg x hr/mL)	Internal dose (mg/kg)
Foetus (30 mg/kg po single dose, actual)	0.06	0.72	15
Foetus (30 mg/kg po single dose, simulated)	0.06	1.05	15
Foetus (30 mg/kg po repeat 9 daily doses)	0.08	-	-
Foetus (400 mg/kg po single dose, simulated)	0.24	5.99	84
Foetus (1000 mg/kg po single dose, simulated)	0.32	8.68	120

2.*PPO inhibition/PPIX accumulation:* Using PPIX accumulation as a marker for PPO inhibition, there was a correlation between PPIX accumulation and developmental toxicity, as demonstrated by flumioxazin-related PPIX accumulation in the rat and not the rabbit foetus and demonstrated compound-specific differences.

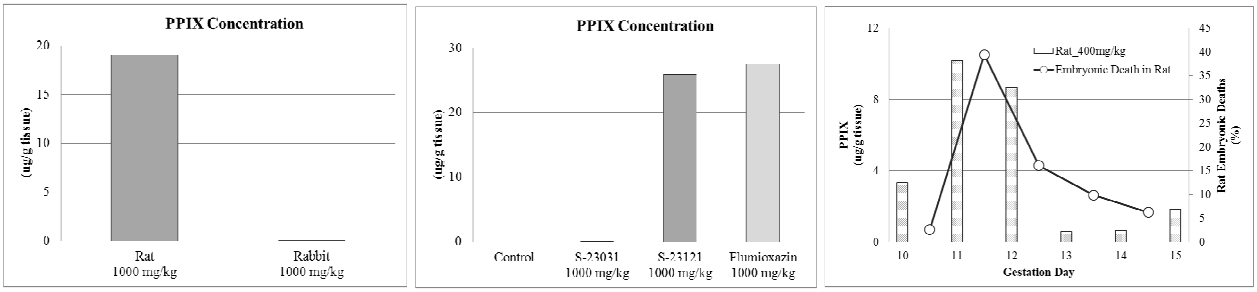


Fig. 3 Close link between PPIX accumulation and developmental toxicity

Sensitivity to inhibition was not different between rat adult and foetal PPO.
The induction ratios of PPIX following treatment with flumioxazin at 0.3 µg/mL were 10.3, 4.4, 1.1 and 1.4-fold in primary hepatocytes of rat, human, rabbit and monkey, respectively (rat>human>rabbit/monkey). Based on IC_{50} values, the relative sensitivity of the species to PPO inhibition by flumioxazin was rat > human > rabbit.

3.*Pathogenesis of the developmental effects*: Comparison of the pathogenesis of the developmental effect of flumioxazin between rats and rabbits (GD 12, 1000 mg/kg), identified abnormal iron deposits in the mitochondria, probably due to inhibition of haem biosynthesis, in polychromatophilic erythroblasts that were observed as early as 6 hours after treatment, in rat foetuses. Subsequent degeneration of these

erythroblasts was indicative of foetal anemia. Histological examination of hearts from exposed embryos revealed thinning of the ventricular wall by 36 hours after treatment. This may reflect compensation for the loss of embryonic blood cells. Therefore, the VSD caused by flumioxazin appears to result from inhibition of haem biosynthesis rather than from direct injury to embryonic heart tissue. Observations in the pathogenesis of developmental effects of flumioxazin in rat foetuses included: anemia, reduced serum protein, enlarged heart, oedema, delayed closure of the interventricular foramen, and incomplete/ delayed ossification of the ribs. Enlarged heart is seen among surviving foetuses in concurrence with severe foetal anemia suggesting that enlarged heart results from pumping greater volumes of blood in compensation for foetal anemia. Enlargement of the heart precedes interventricular foramen closure. Therefore, the VSD caused by flumioxazin may be due to failure of heart closure resulting from mechanical distortion of the heart or abnormal blood flow rather than from direct toxic effects of flumioxazin on cardiac tissue. Reduction of foetal serum protein due to reduced production in the liver in response to hypoxia, was considered to lead to incomplete/delayed ossification of the ribs and the wavy ribs seen at term. None of these effects were apparent in rabbit foetuses.

New mechanistic data:

These studies were conducted after the publication of the current Flumioxazin classification in the 28th ATP of the DSD. The purpose was to add weight to the mechanistic evidence and to allow assessment of the human relevance of the developmental toxicity found in rats.

Method	**Result**	**Reference**
Rat pharmacokinetic study (3 females/gp) oral: gavage (Crl:CD (SD): 1000 mg/kg/ 3.7 MBq/single dose Non guideline non-GLP	The total amounts of ^{14}C excreted into bile and urine and ^{14}C which remained in the carcass showed that the absorption (bile + urine + carcass) in females was 12.3% after a single oral administration of flumioxazin at 1000 mg/kg.	Takaku, T. (2012a) SBM-0092
Rat / Rabbit pharmacokinetic study (4 females/gp) oral: gavage Rat (HW): 30 mg/11.3 MBq/5 mL/kg/d rabbit (NZW): 30 mg/1.12 MBq/0.5 mL/kg/d (exposure from GD 6 – 12) Japanese guidelines on non-clinical pharmacokinetic studies no.496; non-GLP	In rats, significantly higher transfer of radioactivity to blood cells was observed compared with rabbits. Elimination of radioactivity from female reproductive tissue of both species was slower than that from plasma, with only a small amount of radioactivity being transferred to the fetus.	Shirai, N. (2009) SBM-0081
Development of rat erythroblasts in rat embryos, *ex vivo* rat (SD) male/female No test material added, study used to examine differentiation of developing erythrocytes No guideline available; non-GLP	In rats differentiation of circulating erythroblasts in rat embryos from embryonic day 11 to 14 was synchronised.	Ihara, R. (2011) SBT-0117
Flumioxazin/metabolites inhibition of PPO obtained from rat liver mitochondria,	Flumioxazin has the strongest inhibitory activity among the 4 substances tested, followed by	Abe, J. (2011a) SBT-0118

in vitro S-53482: 10 pM - 1 µM 3-OH S-53482: 100 pM - 10 µM 4-OH S-53482: 1 nM - 100 µM APF: 1 nM - 100 µM No guideline available; non-GLP	3-OH S-53482 and 4-OH S-54382, which were 13.7 and 147 times weaker than flumioxazin. APF does not have any inhibitory activity against PPO up to 100 µM.	
K562 cell differentiation into erythroid cells in the presence of *flumioxazi*n, *in vitro* 0.01 - 5 µM No guideline available; non-GLP	PPIX accumulation in K562 cells was observed at concentrations of 1 µM and greater in dose dependent manner, there however was no effect on cell proliferation or haem synthesis at the highest dose tested.	Kawamura, S. (2012a) SBT-0119
K562 cell differentiation into erythroid cells in the presence of *metabolites of flumioxazin, in vitro* 5 µM No guideline available; non-GLP	There was no effect on protoporphyrin IX content, heme synthesis and cell proliferation when K562 cells were treated with the metabolites, while flumioxazin increased protoporphyrin IX in K562 cells.	Kawamura, S. (2012b) SBT-0123
Species difference in accumulation of PPIX in primary hepatocytes from rat, rabbit, monkey & human, *in vitro* 0.01 - 0.3 µg/mL No guideline available; non-GLP	The induction ratios of PPIX following treatment with flumioxazin at 0.3 µg/mL were 10.3, 1.1, 1.4 and 4.4-fold in primary hepatocytes of rat, rabbit, monkey and human respectively. These results suggest that rat hepatocytes are more sensitive to flumioxazin treatment than the other 3 species, including human.	Abe, J. (2011b) SBT-0120
PBPK modelling of flumioxazin in rats and humans, *in vitro* and *in silico* 5.6 – 100 µM No guideline available; non-GLP	The developed human PBPK model demonstrated that the human fetal exposure to flumioxazin following a maternal oral dose of 1000 mg/kg would be 0.68 ppm (1.92 µM), indicating that exposure to flumioxazin in a human fetus would be relatively low, even at 1000 mg/kg.	Takaku, T. (2012b) SBM-0093

The following information was taken from these studies

1. The absorption of flumioxazin following a single high oral dose (1000 mg/kg) was 12.3% (bile+urine+carcass). The internal dose is therefore 124 mg/kg.

2. Significantly higher transfer of total radioactivity to blood cells was observed in rats compared with rabbits. Elimination of total radioactivity from female reproductive tissue of both species was slower than from plasma, with only a small amount of radioactivity being transferred to the foetus. However, radiolabelled flumioxazin was detected at a concentration of 0.02 µg eq./g in both rat maternal plasma and foetus, therefore, there was no difference in exposure to flumioxazin between maternal animals and fetuses.

3.The relative sensitivity of the rat foetal erythroblast to flumioxazin induced anaemia was attributed to the observation that in rats, differentiation of circulating erythroblasts in the embryos from embryonic day 11 to 14 was synchronised (see argument 2 below).

4.Additional *in vitro* studies using human (erythroleukemia-derived K562 differentiation and haem production) and rat (erythroleukemia-derived cells) cell lines were conducted to support the proposal that human erythroid cells are less sensitive to the effects of flumioxazin (*via* on PPO) on haem production that rat.

5.Finally, a PBPK model was developed for flumioxazin in order to predict parent flumioxazin concentrations in blood and foetus of pregnant humans based on data obtained in the rat. An *in vitro* metabolism study using rat and human liver microsomes was carried out to determine any species differences in the metabolism of flumioxazin between rats and humans. Physiological data for humans were cited from the literature and the human model was developed to predict the pharmacokinetics in humans in several tissues. Whilst it is not possible to measure experimentally human foetal concentrations of flumioxazin, development of the PBPK model in the pregnant rat was scaled to humans to provide an estimate of the disposition of flumioxazin in pregnant humans. The human pregnant PBPK model developed demonstrated that the human foetal exposure to flumioxazin following a maternal oral dose of 1000 mg/kg would be 0.68 ppm (1.92 µM).

In addition, the results of the developmental toxicity studies which were carried out in the rat and rabbit on two structural analogues of flumioxazin, (one causing developmental toxicity and the other not) was correlated with ability of these analogues to inhibit PPO/induce accumulation of PPIX in mitochondrial preparations of treated rat and rabbit livers.

Further developmental mechanistic studies conducted on Flumioxazin and its metabolites demonstrated that flumioxazin metabolites had no effect on PPIX accumulation, haem synthesis or cell proliferation in K562 cells.

Reference was made to the published data on x-ray irradiation (Wilson et al. (1953)) and nimustine (Miyagawa et al. (1988)). Both have been shown to produce VSDs most likely via direct damage to the heart as a result of their ability to damage cardiomyocytes. The critical period for cardiac damage by these agents is determined to be earlier than GD 12. This was considered to support the theory that flumioxazin might not produce cardiac VSD via direct damage to embryonic heart tissue but rather through an indirect mechanism.

The case for declassification relies mainly on two arguments;

1.<u>Species specificity in sensitivity to PPO inhibition</u>

Comparison of IC_{50s} (nM) among three species for PPOs derived from adult liver

Species	rat	human	rabbit
IC_{50}	7.15	17.3	138

As shown in the table above, rat PPO was most sensitive to inhibition by flumioxazin among the species tested while humans were intermediate between rats and rabbits. Consistent with this finding is the observation that significant accumulation of PPIX as the result of PPO inhibition in (primary) hepatocytes was observed in rats with a smaller amount of PPIX detected in human hepatocytes.

PPO inhibition causes anaemia which is the primary toxic effect in rats both in adults and embryos. This anaemia is considered to be responsible for the developmental toxicity in the

rat. In humans, variegate porphyria (VP) is a disease associated with PPO deficiency. VP is associated with reduced PPO content and delta-aminolevulinic acid dehydratase ()activity in erythrocytes. Erythrocyte counts are not affected in VP and haemoglobin, haematocrit, MCV and MCH in VP are slightly higher than their controls. The reduced rate of haem production in VP is enough to generate the same, or even greater, quantity of haemoglobin as control individuals. This indicates that PPO activity is not rate-limiting in human erythroid cells and almost normal haem concentration (without anaemia), can be maintained even with reduced PPO activity. In contrast, PPO activity is close to rate-limiting in rat erythroid cells and decreased activity reduces porphyrin production in erythroids resulting in PPIX accumulation, iron deposit, and anaemia. Studies conducted with K562 (human erythroleukaemia derived), CD36+ (human cord blood cells) and a rat erythroleukaemia cell line indicated that although PPIX accumulation (therefore PPO inhibition) occurred in the human cell lines, there was no effect in haem content or the number of differentiated haem-synthesising cells when treated with flumioxazin from 0.01 to 5 µM, while haem production was reduced in the rat cell line in response to flumioxazin.

A PBPK model for flumioxazin was developed to predict flumioxazin concentration in the maternal blood and foetus of pregnant human. The model predicted that the flumioxazin concentration in the human foetus at a dose of 1000 mg/kg po was 0.68 ppm (1.92µM). This concentration is lower than the maximum no effect concentration of 5 µM in K562 cells and was considered to support the view that humans would be less susceptible to anaemia and the developmental effects of flumioxazin.

2. Synchronous maturation of rat erythroblasts.

It is proposed that rat embryos are particularly sensitive to haematotoxic effects of flumioxazin due to the synchronous differentiation of erythroblasts, whereas a relatively heterogenous population is reported to occur in human embryos during primitive haematopoiesis. The morphology and population characteristics of blood cells in rat embryos demonstrated that a vast majority of erythroblasts are polychromatophilic on GD 12, the day of the greatest sensitivity, and orthochromatophilic erythroblasts on gestational day 14, when rat embryos were much less sensitive to flumioxazin.

This is thought to explain why flumioxazin induces an enormous and synchronous loss of blood cells in rat embryos exposed to flumioxazin. Synchronised differentiation of erythroblasts in rat embryos does not allow for an effective compensation of haem synthesis inhibition in the critical gestational days 12 to 14. Fresh blood cells would not be supplied until haematopoiesis shifts from the yolk sac to the liver (GD 17). In humans, erythroblast formation in the yolk sac is characteristic for embryonal days 20 – 50 and is extended over a period of several weeks; haematopoiesis then shifts to the liver and finally to the bone marrow. In contrast to rats, a relatively heterogeneous population was observed in human primitive haematopoiesis; three types, with the possibility of differing sensitivities to flumioxazin. This relative sensitivity of the human embryonic erythroblast populations has of course not, however, been demonstrated. The DS conceives that it is possible that the type III erythroblast corresponds to the orthochromatophilic erythroblast, and type I and type II correspond to earlier erythroblasts, presumably basophilic or polychromatophilic. Relative populations of type I, II, and III observed in the yolk sac range from 7% to 40%, from 21% to 89%, and from 4% to 65%, respectively, during the period from commencement of human primitive haemopoiesis in week 3-4 to completion of ventricular septum formation in week 8. Thus it is surmised that in humans, even if a particular population is lost, blood cell loss could not be as massive as in rats.

Overall, it was concluded by the DS that the rat is an inappropriate model for assessing the developmental toxicity of flumioxazin in humans because, unlike humans, they are highly sensitive to PPO inhibition, resulting in foetal anaemia and consequent developmental toxicity. The DS considered that the species difference in susceptibility to flumioxazin and potential for

anaemia was sufficient support for the removal of the Cat 1B reproductive toxicity classification, on the basis that there was no plausible scenario whereby humans would be at risk of developmental toxicity.

Comments received during public consultation

Five MS opposed the proposed declassification. A number of MS suggested that the proposal shouldn't be accepted without an in-depth analysis of the argument and it was proposed that classification in Cat 2 should be assessed. There were six comments from individuals in the US and one from the UK supporting the declassification proposal. The proposal was supported by industry.

In addition, industry submitted two new study reports using human CD36+ cells and rat erythroleukemia cells.

1. CD36+ cells are precursor of erythroblasts, which can be differentiated into haem-synthesizing cells and are considered more relevant to physiological maturation of human foetal erythroblasts than K562 human erythroleukemia cells, hence better addressing the effect on haem biosynthesis in human foetal erythroid cells. There were no effects on haem content and cell number of human haem-synthesizing cells treated with flumioxazin at up to 5 µM.

2. The rat erythroleukemia cell line which can be differentiated into haem-synthesizing cells by treatment with inducers and can correspond to human K562 cells. The results revealed that flumioxazin at 0.1µM and above reduced haem production in the rat haem-synthesizing cells. These data were considered by the notifier and the DS to support the case for declassification.

Additional key elements

The CLH report included an updated discussion on the human relevance of the developmental effects included by flumioxazin in rats (Annex 1) which used a Human Relevance Framework (HRF)-type of analysis of the data set. The proposed mechanism is discussed in the light of all evidence presented and other potential modes of action are considered. The critical biological events and their relevance to humans are discussed. The conclusion is that the human embryo would be far less sensitive than the rat embryo to the effects of flumioxazin for the following reasons:

1. Adult human PPO is significantly less sensitive to flumioxazin than rat PPO *in vitro* and at the cellular level.
2. Decreased PPO activity in rat erythroid cells results in anemia leading to developmental toxicities. In contrast, reported clinical evidences demonstrate that PPO activity is much higher than a rate-limiting enzyme in the haem synthesis pathway in human erythroid cells. It is therefore considered to be unlikely that the reduction of PPO activity could induce anemia or disturbance of haem synthesis in human erythroid cells.
3. Human erythroblasts are considered to be non-susceptible to flumioxazin when treated at concentrations that are expected to far exceed those attained in human embryos following flumioxazin exposure. Pharmacokinetic modeling in the rat and the human predicts that human erythroblasts would not be susceptible to flumioxazin at exposures exceeding a maternal dose of 1000 mg/kg/d.
4. Because of a less homogeneous population in primitive erythropoiesis in humans, it is considered possible that a loss of a particular population should not lead to a massive drop in red cells.
5. Rat erythrocytes are more fragile than human erythrocytes, for example when exposed to osmotic imbalance, pH changes or oxidative damage.

Assessment and comparison with the classification criteria

Flumioxazin induced embryolethality and teratogenicity in the rat following dosing *via* both the oral and dermal routes. Abnormalities induced were cardiovascular, mainly VSD, and an increase in the incidence of wavy ribs and reduced ossification of sacrococcygeal vertebral bodies were also observed. Furthermore, foetal growth retardation was also observed in both studies. This observation was supported by the occurrence of reduced litter size (embryo-foetal lethality) and reduced pup weight seen in the 2-generation study. These effects were observed in the rat at relatively low levels and in the absence of maternal toxicity. In contrast to the rat, flumioxazin showed no evidence of developmental toxicity in the rabbit even in the presence of maternal toxicity. The maximum dose administered in the rabbit study was 100-fold greater (3000 mg/kg/d) than the maximum dose administered in the rat oral developmental study.

The applicant for PPP authorisation has carried out an extensive program of research (described in some detail above) with flumioxazin in an effort to elucidate the mechanism of the developmental toxicity seen in rats. Existing mechanistic studies, which were evaluated during the previous review of flumioxazin for inclusion in Annex 1 to DSD, have been supplemented by additional studies to strengthen the mechanistic case and to allow an assessment of the relevance to human of developmental effects found in rats.
There is reasonable evidence for a single mode of action causing the developmental toxicities in the rat. The sequence of key biological events in the proposed mode of action has been investigated. According to the proposed mechanism, inhibition of PPO interferes with normal haem synthesis, which causes loss of blood cells leading to foetal anaemia, embryolethality and the development of malformations.
Rats are particularly sensitive to the effects of PPO inhibition induced by flumioxazin in erythroblasts. This leads to anaemia which is considered to be a critical precursor of the developmental toxicity resulting from flumioxazin exposure. In contrast, humans may be less likely to develop anaemia from PPO inhibition. This conclusion is based on (1) clinical findings that PPO deficient patients with VP show no signs of anaemia, (2) experimental evidence that flumioxazin does not reduce haem production in K562 cells/CD36+ human erythroid cells and (3) that humans are less sensitive to PPO inhibition than rats.
Pharmacokinetic modeling in the rat and the human predicts that human erythroblasts would not be susceptible to flumioxazin at exposures equivalent to a maternal dose exceeding 1000 mg/kg/day, and this is considered to support the species difference in sensitivity.

<u>*Comparison with the criteria*</u>
The data from the rat developmental toxicity studies, both oral and dermal, clearly conform to the criteria for classification in Cat 1B;
…clear evidence… based on animal data …of an adverse effect on development in the absence of other toxic effects.

However, the DS has provided plausible evidence for a single mode of action (described above) to explain the embryo-foetal toxicity and teratogenicity. Mechanistic studies have addressed the biological events in the proposed mode of action, whereby foetal anaemia and adverse effects on the cardiac system may be induced *via* inhibition of PPO. While the proposed mode of action is considered plausible by the RAC, there is still some doubt, as the postulated sequence of events supporting the mechanism has not been demonstrated in the rat at the dose level of 30 mg/kg. Further data have explored the relative sensitivities to flumioxazin toxicity in rats, rabbits and humans and whether the rat is a relevant model in the assessment of the hazard in humans. The DS assessed the effects to be not relevant to humans and on the basis of the criteria, that classification is not appropriate.

Regulation (EC) No 1272/2008:
„The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or

on development 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 other toxic effects. However, when there is ***mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate****."*

On the basis that species sensitivity may raise some doubt about the relevance to man by the data presented, RAC agrees that classification in Cat 2 for developmental toxicity could be considered.

However, the DS proposal is to remove the classification as Repr. 1B, resulting in no classification. The DS believes that the sensitivity of man to this mechanism is likely to be low (less PPO inhibition/PPO not rate limiting in haem generation in humans/non-synchronous maturation in human embryonic erythroblasts/low quantities of chemical estimated to reach the foetus), and therefore the risk of this hazard occurring is negligible. They state the following in the CLH report:

'Consequential avoidance of the quantitative aspect in criteria of hazard (for man) may be justified from the procedural point of view. On the other hand, when the **risk for humans is negligible**, should hazard identification and characterisation neglect this fact completely?

This question from the DS raises the central element of the rationale for removing the classification as Repr. 1B. It is not ruled out that the hazard also potentially exists for humans, notwithstanding the possible interspecies differences in PPO sensitivity and the particular sensitivity of the rat foetus to induction of anaemia through this route. The issue is whether the element of risk should be considered relevant to classification and if so, has this risk been proven to be negligible?

The DS contends that the argument for 'no classification' is supported by the criteria in the CLP Regulation, which states that:"*If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the* ***toxicokinetic differences*** *are so marked that* ***it is certain*** *that the hazardous property will not be expresssed in humans then a substance which produces an adverse effect on reproduction in experimental animals should not be classified.*"

RAC does not agree that the mechanism has no relevance to humans and does not agree that declassification is appropriate on the basis of this argument.

Toxicokinetics determine the rate at which a chemical is absorbed into the body and distribution/ metabolism/ elimination thereafter. RAC is not of the view that the differences identified in the data set are 'toxicokinetic' other than the outcome of the PBPK modelling which suggests that the low levels of flumioxazin in the foetus following a maternal dose of 1000 mg/kg would not cause foetal anaemia. It is noted that the exposure level in the rat foetus following a maternal dose of 30 mg/kg is measured at approximately 0.02 µg eq./g indicating extreme sensitivity of the (rat) foetus to flumioxazin. Also, the same effect occurred in the rat following *dermal* maternal exposure to 300 mg/kg with unknown foetal exposure (dermal penetration approx. 10%). The observation that rat, human, rabbit and monkey hepatocyte PPO is inhibited to different degrees *in vitro*, and that PPO activity is not rate-limiting in human haem synthesis are not toxicokinetic aspects of flumioxazin toxicity. The main argument concerning the relative susceptibility of the rat foetus to anaemia due to its synchronously differentiating erythroblasts is also not a toxicokinetic difference and while plausible, it is not proven that no significant damage will occur in the human foetus.

RAC considers that the data provided **do not support this argument for non-classification**, i.e., that *the* ***toxicokinetic differences*** *are so marked that* ***it is certain*** *that the hazardous property will not be expressed in humans.*

The differences are not considered to be solely toxicokinetic; some are qualitative (erythrocyte maturation/reasons for different PPO inhibition) some quantitative (PPO activity not rate-limiting in humans/PPO inhibition rate). Overall, it is **not certain** that the risk is negligible. The proposal to remove the classification on the basis of this criterion is not supported.

Specific points considered by the RAC

1. It is noted that the developmental effects are seen in the rat at 30 mg/kg bw/day in the oral study in the absence of signs of maternal toxicity. Effects seen in female rats following 13 weeks exposure (sub-chronic studies) at this dose level were slight but significant changes in the haematopoiesis and included the following: moderate decreases in haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and haematocrit values at 300 ppm (approx. 20 mg/kg bw). The occurrence of anaemia in the dam at this dose level is likely to be minimal. However, the adult erythrocyte is likely to be significantly less sensitive to flumioxazin than the primitive erythroblast of the early rat embryo. The foetus at this dose may have suffered sufficient anaemia to cause the severe malformation of the heart and other developmental toxicity. It is noted that in the rat/rabbit pharmacokinetic study (Shirai, 2009; SDM-0081) that after the initial dose, Cmax/min of ^{14}C concentration in plasma ranged from 4.49 to 0.70 in rats and that most of the previous dose of ^{14}C was excreted before the next dose. This pharmacokinetic study indicates that flumioxazin derived radioactivity was detected at a concentration of 0.02 µg eq./g in both maternal plasma and foetus. Therefore, there was no difference to exposure to flumioxazin between maternal animals and foetuses. This indicates extreme sensitivity of the rat foetus. As the amount of flumioxazin in both foetus and maternal blood (7 hours after dosing with 30 mg/kg) is the same and the degree of inhibition of PPO is also similar, the proposed sensitivity of the rat foetus relies on the particular sensitivity of rat embryonic erythroblast development at the critical window of exposure as discussed by the DS (synchronous maturation of erythroblasts in rats etc).

   The adverse effects are seen *at 30 mg/kg* in the developmental toxicity study. Considering the critical window study (single critical day: day 12) and the pharmacokinetic study, these effects (in the developmental toxicity study) could have occurred following a single dose of approximately 30 mg/kg (internal dose 15 mg/kg), while the studies to explore the pathogenesis of these effects used a single dose of 400 mg/kg (internal dose possibly 84 mg/kg) and 1000 mg/kg (internal dose 124 mg/kg). The internal dose at 400 and 1000 mg/kg would be 5.6 times and 8 times higher respectively, than at 30 mg/kg. However, the effects seen in the developmental toxicity study are similar/identical to those induced following a single albeit much higher, dose.

2. PPO inhibition by flumioxazin was investigated using cryopreserved hepatocytes of rats, rabbits, monkeys and humans (Abe, 2011). The results show that no PPIX accumulation was observed in rabbit and monkey hepatocytes at the maximum tested concentration of flumioxazin. Remarkable accumulation of PPIX as the result of PPO inhibition in hepatocytes was observed in rats with a smaller amount of PPIX detected in human hepatocytes. These results show that human PPO is less sensitive to flumioxazin than rat PPO even at the cellular level, reflecting an *in vitro* inhibitory potency of flumioxazin. The reason for the apparent interspecies differences in PPO sensitivity demonstrated in hepatocytes *in vitro* is not known. PPO is a highly conserved enzyme, with no information published about isoforms within or between species. The difference (in IC_{50}) between humans and rats is also not great (approx. 2.4). This is an argument about reduced risk but not for lack of hazard.

3.In order to investigate whether or not PPO inhibition in erythroblasts can cause anaemia in humans, the DS compared resistance to the disturbance of haem synthesis and induction of anaemia by flumioxazin-induced PPO inhibition in human and rat erythroid cell lines (K562 (Kawamura, 2011), CD36+ (Kawamura, 2013a), and REL cells (Kawamura, 2013b)). These cell lines are used as a model for human and rat erythroid maturation since the cells can be differentiated into haemoglobin-synthesizing cells. *In vitro* data generated in K562 cells (human erythroleukemia), rat erythroleukemia cells *and* human cord blood (CD36+) cells, demonstrated that flumioxazin did not interfere with erythroblast differentiation, proliferation and haem production in human cells whereas rat cells were adversely affected. This evidence was considered to support the proposal that reduced PPO activity becomes rate-limiting in the rat but not in human erythroid cells.

There are however, some uncertainties involved in extrapolation of such data to the *in vivo* human foetus. For example, does this *in vitro* assay with human cell lines relate closely to the transient population of human embryonic erythroblasts in early embryo/foetal development?

4.There is certainly no doubt that the rat embryo is very sensitive to flumioxazin. The DS considers that this is due to the synchronous maturation of the erythroblasts in the rat embryo yolk sac and that this is not likely to occur in the human embryo because of the significantly greater capacity for the human foetus to recover (as discussed above). This is plausible, but not proven. Also, even if the human foetus erythroblasts populations occur in different ratios throughout a relatively long period and the yolk sac erythropoiesis is relatively short, exposure to flumioxazin is likely to cause some damage, the nature of which and rate of recovery from which are still unknown.

A.Mechanism of action

1. The mechanism is considered plausible on the basis of the following supporting data:

2. -demonstration of a critical window for all developmental toxicity endpoints

3. -correlation between inhibition of PPO (via PPIX accumulation) and developmental toxicity *via* comparison of rat and rabbit and compound-specific differences between flumioxazin and chemical analogues.

4. -histological evidence of mitochondrial lesions in polychromatophilic erythroblasts and cardiac damage in the rat foetus (compared to no effect in rabbits).

5. -demonstration of the pathogenesis of the developmental effect in rat (no effect in rabbit).

6. **However**, there are some remaining uncertainties;

7. - the critical events were observed at significantly higher doses than in the developmental toxicity study and were not demonstrated in the rat foetus when the dams were dosed with 30 mg/kg bw .However, it is not uncommon that a single higher dose is necessary to produce the developmental toxicity which is similar to those produced by multiple doses. Considering a decrease in absorption rate with each incremental increase in the oral dose, differences in internal dose levels would not be as large as those calculated from dose levels administered orally. The internal dose of 400 mg/kg is 84 mg/kg and becomes 5.6 times higher than that of 30 mg/kg (internal dose 15 mg/kg). The internal dose for 1000 mg/kg is approximately 124 mg/kg. The stated purpose of using the high dose of 1000 mg/kg (Kawamura et. al., 1996) was to produce obvious, initial histological changes related to VSD in most of the embryos examined because a single

administration of 400 mg/kg was shown to cause the effect in 14% of surviving foetuses in a preceding study (SBT 0065). Pharmacokinetic studies indicated that the maternal plasma and foetal flumioxazin concentration after a single dose of 30 mg/kg was very low (0.02 µg eq./g) and each dose was almost completely cleared within a 24 hour period (indicating little potential for accumulation/addition).

8. - In addition, the PPO inhibition data indicate similar IC_{50} values for adult and foetal rats (ref study SBT-0045), yet the foetus was far more sensitive to flumioxazin damage. In contrast, PPIX was demonstrated to accumulate in the rat foetus at very high levels compared to adult liver, which could be due to rapid excretion into bile and faeces in the adult.

9. - Placental transfer data and the rat pharmacokinetic study (Shirai, 2009) indicate flumioxazin derived radioactivity was detected at a concentration of 0.02 µg eq./g in both maternal plasma and foetus following a dose of 30 mg/kg. Therefore, there was no difference in exposure to flumioxazin between maternal animals and foetuses. This points to extreme sensitivity of the rat foetus, believed to result from the non-synchronous maturation of rat embryonic erythroblasts and the potential rate-limiting role of PPO inhibition in haem synthesis in the rat.

10. -The mechanism proposed is likely to occur in humans, even though the evidence suggests that human erythroid-forming cell lines (K562/CD34+) are more resistant to flumioxazin induced inhibition of haem synthesis than rats and that PPO activity is not rate limiting in humans but significantly rate-limiting in rats. The difference is, however, generally more quantitative than qualitative.
11.

12. B. Human relevance:

13. The human relevance of the mechanism is questioned (by the DS) on the basis that:

14. -A clear species difference is demonstrated between rats and rabbits with respect to the developmental toxicity induced by flumioxazin. This is supported by the lower sensitivity to PPO inhibition and PPIX accumulation in rabbits.

15. It is noted that the difference between rats and humans with respect to PPO inhibition is, however, less marked. For example, IC_{50} values measured were 0.007, 0.017, 0.138, in liver mitochondria from rats, humans and rabbits, respectively. PPIX induction in primary hepatocytes was 10.1, 4.4, 1.1, and 1.4 fold in rat, human, monkey and rabbit, respectively. Human cells were closer to rat than rabbit with respect to IC_{50} and closer to rat than rabbit and monkey with respect to PPIX accumulation. The reason for the differences in species sensitivity to flumioxazin induced PPO inhibition is not known.

16. -It is proposed that the human foetus will be less sensitive to anaemia induced erythroblast damage than the rat due to the synchronous maturation of the erythroblasts and the sensitivity of polychromatophilic erythroblasts on a single day (12) of gestation in rats.

17. -It was demonstrated that human erythroid cell lines (K562/CA36+) could differentiate normally in the presence of flumioxazin and that haem production was not affected even though PPO inhibition occurred. PPO inhibition was associated with reduced haem in a rat REL cell line.

18. **However,** the demonstrated difference (PPO inhibition 2.4 –fold) between rat and human cells is not large and represents a moderate quantitative difference.

19. -Even though rat embryos may be particularly sensitive to anaemia induced by destruction of polychromatophilic erythroblasts in day 12, it is not known what the effect may be in human embryos during the sensitive period of rapid haem synthesis and

differentiation of the primitive erythroblast.

20. Note: The antimalarial artemisinins target embryonic erythroblasts and maternal reticulocytes (via damage to haem producing mitochrondia (and by altering the cell cycle (Finaurini, S., et. al. 2012)) causing embryotoxicity and maternal reticulocytopenia in rats. The authors concluded that the therapeutic dose range causing maternal reticulocytopenia in pregnant women is associated with a risk of adverse effects on the embryo (Clark et al.,, 2011). These authors have previously reported developmental toxicity and teratogenicity of artemisinins in rats at doses causing only a 15% reduction of maternal reticulocytes, ie., embryos are more sensitive and also concluded in the previous paper (Clark 2009) that ..'doses in (human) pregnancy during the sensitive period (post conception day 21 to week 9) which might cause even a minor decrease in adult reticulocyte count could cause a marked depletion of embryonic erythroblasts which could lead to death or malformation of the embryo'. It is noted that the adverse effect on embryonic erythroblasts was also reported in a primate study (Clark et al., 2008

21. The mechanism of toxicity is not the same for flumioxazin but the target cells appear to be, therefore the sensitivity of both rat and human embryonic erythroblasts is relevant.

22. Conclusion

23. RAC considers that the justification for removing the classification is not adequate While taking account of all arguments related to relative sensitivity of the rat, significant doubt still exists as to the actual sensitivity of the human foetus to PPO inhibition during a sensitive period of erythrocyte maturation. The mechanism of flumioxazin-induced developmental toxicity is considered relevant to man, although it is acknowledged that significant differences between rat and man may exist with regard to sensitivity to this mechanism for the reasons outlined above. **The RAC concludes that the doubts with regard to human relevance are not sufficiently convincing to warrant classification as Repr. 2 or no classification and that the current classification for flumioxazin as Repr. 1B should be retained.**

4.13 OTHER EFFECTS

4.13.1 NON-HUMAN INFORMATION

4.13.1.1NEUROTOXICITY

Not relevant to this proposal.

4.13.1.2IMMUNOTOXICITY

Not relevant to this proposal.

4.13.1.3 SPECIFIC INVESTIGATIONS: OTHER STUDIES

Not relevant to this proposal.

4.13.1.4HUMAN INFORMATION

None.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Summary of the Dossier submitter's proposal

Flumioxazin currently has a harmonised classification as Aquatic Acute 1 (M-factor=1000) and Aquatic Chronic 1 according to CLP. The dossier submitter (DS) carried out the environmental hazard assessment in order to determine the chronic M-factor, currently not included in Annex VI of the CLP Regulation, taking into account the new criteria brought in by the 2nd ATP to CLP and which are related to the classification of long-term hazards to the aquatic environment.

Degradation

Two hydrolysis studies according to guideline EPA-FIFRA 161-1 and in compliance with GLP were run at pH 5, 7 and 9 at 25 °C for 30 days. Flumioxazin was rapidly hydrolysed in all three buffered solutions and the degradation rate increased with pH (DT_{50}: 3-5 d at pH=5, 19-26h at pH=7, 14-23min at pH=9). Degradation proceeded via opening of the cycloimide ring at all pH values to form 482-HA (7-fluoro-6-[(2-carboxyl-1-cyclohexenoyl)amino]-4-(2-propynyl)-1,4-benzoxazin-3-(2H)-one). Subsequent cleavage of the amide linkage to form APF (6-amino-7-fluoro-4-(2-propynyl)-1,4-benzoxazin-3-(2H)-one) and THPA (3, 4, 5, 6-tetrahydrophthalic acid) was observed only at pH 7 and 5. A supportive hydrolysis study performed with flumioxazin and its degradation product 482-HA showed that the hydrolysis of flumioxazin proceeds predominantly through neutral and base catalyzed processes, while the hydrolysis of 482-HA proceeds predominantly through an acid catalyzed process. Half-lives of 482-HA were calculated to be 2.35 hours, 10.7 days and 72 days at pH 5, 7 and 8, respectively.

The photodegradation of flumioxazin in water was studied according to guideline EPA-FIFRA 161-2. The two studies, in compliance with GLP, were carried out at 25 °C and pH 5.0 for 30 days. Light slightly enhanced degradation of flumioxazin in water at pH 5 and a different degradation pathway was involved. The DT_{50} of flumioxazin was 21 h in the light, while DT_{50} in the dark was 118h. 482-PHO (N-(2-propynyl)-4-[4-carboxy-3-fluoro-2-(3,4,5,6-tetrahydrophthalimido)-2-butenylidene]azetidine-2-one) and THPA were identified as major photolytic degradation products.

A ready biodegradability test was performed according to OECD guideline 301 B. The study was carried out in compliance with GLP at 22°C for 28 days using as the inoculum an activated sludge not previously knowingly exposed to the test substance. Biodegradation of flumioxazin was 3% at the end of the test on day 28 so the substance is considered not readily biodegradable under the conditions of the test.

A water/sediment simulation study, carried out according to SETAC guideline, using radio-labelled flumioxazin was run for 98 days at 20 °C using two systems (clay loam, 8% OC and sandy clay loam, 3.6% OC). Flumioxazin was temporarily found in sediment (max 27% after 7d) and it rapidly disappeared in both water and sediment phases. For the whole systems, DT_{50}s were < 1.85d and DT_{90} were 25-69d. Degradation occurred via hydrolysis to APF (max 58% in water after 7 d) and THPA (max 63% in water and 18% in sediment after 7d). Non-extractable residues reached averages of 38-61% and mineralisation reached averages of 5-29% after 98 days. The available water sediment study has been reassessed using FOCUS kinetics approach, and as no reliable $DegT_{50}$ values have been obtained for water and sediment, the geometric mean value of 21.6 days was used for the water phase and the default value of 1000 days was used for the sediment phase.

A further water/sediment study (Shibata, 2011) was carried out according to OECD Guideline 308. The study was run for 30d and two systems were set up containing natural sediment and associated water and suitable traps for collecting volatile compounds. The degradation rate of

flumioxazin in water in the absence or presence of sediment or light, is largely unaffected, indicating that sediment or light insignificantly contribute to dissipation/degradation.

In this study all major metabolites formed were identified and the presence of sediment decreased the amounts of all metabolites formed. For the whole system and natural water the maximum levels of CO_2 are about 24% and 14.8% (in illuminated conditions) respectively. Moreover, in illuminated water/sediment systems CO_2 and bound residues reached levels ≥48% after 30 days.

Bioaccumulation

The substance has a measured $logK_{ow}$ of about 2.55 (OECD 107, 20 °C, purity 99.5%). The DS did not provide any studies on bioaccumulation.
With a $logK_{ow}$ < 4 the substance does not meet the criterion for bioaccumulation according to CLP.

The 3 major hydrolytic degradation products: 482-HA, APF and THPA have calculated $logK_{ow}$ values of 0.804, 0.127 and 0.88 respectively. The DS indicated that for these data it isn't necessary to carry out a bioaccumulation study in fish.

Aquatic toxicity:

Several acute and chronic aquatic toxicity data are available from studies on the tested substance which, in the majority, followed guideline standards and were in compliance with GLP and reliable according to the DS.

The available short-term tests for flumioxazin were: three for fish, one with invertebrates, three with algae and aquatic plants, respectively. The most sensitive species tested is the aquatic plant *Lemna gibba* (14d semi-static condition test) with an EC_{50}=0.00035 mg/L based on initial measured concentrations, which ranged from 85 to 90% of the nominal concentrations and decreases by 23% at day 3.

The chronic aquatic toxicity of flumioxazin is assessed on the base of three long-term fish tests, four chronic tests with invertebrates and three studies with algae and aquatic plants. The most sensitive species tested was *Navicula pelliculosa* that was exposed to flumioxazin for 120h in static conditions, with the resulting value for NOEC<0.000042 mg/L and EC_5=0.000041 mg/L based on initial measured concentration.

Comments received during public consultation

Four Member States (MS) contributed during public consultation stating a general agreement with the proposed environmental classification.

Two MS had specific comments. They suggested recalculating the EC_{50} value for *Lemna gibba* using data at day 7 (if available) instead of data at day 14 according to OECD test guideline 221. The DS replied that the 7-day EC_{50} is not available.

Concerning the study with *Navicula pelliculosa,* one MS noted that the EC_{50} and NOEC were measured after 5 days, while in OECD guideline 201 for freshwater algae the exponentially growing test organisms were exposed over a period of 72 hours.

Another MS suggested using the value of EC_5=0.000041 mg/L instead of the NOEC from the study with *Navicula pelliculosa*. In this regard, the DS stated that using the EC_5 would influence neither the classification of the substance nor derivation of a chronic M-factor.

A further MS, while agreeing that *Lemna gibba* and *Navicula pelliculosa* were the most sensitive species, highlighted that the EC_{50} and NOEC values are based on the initial mean measured concentration, while it would have been more appropriate to calculate the geometric mean concentration at the start and the end of the test, taking into account that the substance is hydrolytically unstable. This could have an influence on the setting of the M-factor.

The DS stated that according to SANCO 3268/2001/rev.4, the endpoints based on initial measured concentrations are considered relevant when effect data are obtained from the test performed under static conditions.

5.1 Degradation

Table 21: Summary of relevant information on degradation

Method	Results	Remarks	Reference
EPA – FIFRA 161-1, Hydrolytic degradation of [THP-^{14}C]-flumioxazin	Flumioxazin was rapidly hydrolysed in all three buffered solutions and the half-lives were calculated to be 3.43 days, 18.9 - 23.9 hours and 14.0 - 15.1 minutes at pH 5, 7 and 9, respectively.	Rapidly hydrolysed	Katagi et al., 1990a.
EPA-FIFRA 161-1, Hydrolytic degradation of [Ph-^{14}C]-flumioxazin	Flumioxazin was rapidly hydrolysed in all three buffered solutions and the half-lives were calculated to be 4.91 - 5.20 days, 23.2 - 25.9 hours and 21.3 - 22.7 minutes at pH 5, 7 and 9, respectively.	Rapidly hydrolysed	Katagi et al., 1990b.
EPA-FIFRA 161-2, Photodegradation Study with [Ph-^{14}C]-flumioxazin	Light slightly enhances degradation of flumioxazin in water at pH 5 and a different degradation pathway is involved.	None	Fathulla, 1995b
EPA-FIFRA 161-2, Photodegradation Study with [THP-^{14}C]-flumioxazin	light slightly enhances degradation of flumioxazin in water at pH 5 and a different degradation pathway is involved.	None	Fathulla, 1995a.
OECD No. 301 B, Assessment of Ready Biodegradability by Measurement of CO_2 Evolution	Flumioxazin is not readily biodegradable under the conditions of the test. Biodegradation of flumioxazin was 3% at the end of the test on Day 28.	Not readily biodegradable	Graham, R & Alderman, D. (2011)

5.1.1 Stability

Hydrolysis

Conclusion: Flumioxazin is readily hydrolysed in water. Degradation rate increases with pH since DT_{50} values are 3-5 days, 19-26 h and 14-23 min at pH 5, 7 and 9, respectively.

Study 1: Hydrolytic degradation of [THP-^{14}C]-flumioxazin

Guideline: EPA-FIFRA 161-1. Study performed in compliance with GLP. The study is valid.

Cross reference: IIA.7.2.1.1/01, Katagi *et al.*, 1990a.

[THP-^{14}C]-flumioxazin (purity > 99 %), was incubated in sterile aqueous buffered solutions at pH 5, 7 and 9 (0.1 mg/l) for up to 30 days at 25°C, in the dark. Samples were taken at regular intervals throughout the study and were analysed for total radioactivity by LSC. High performance liquid chromatography (HPLC) was used to determine hydrolysis rate and to identify degradation products. Further characterisation of the degradation products was carried out by 2D-TLC with reference standards. The hydrolytic half-lives at each pH were calculated.
The recovery of ^{14}C was 96.4 - 104.9 %. Radioactivity was fully recovered. Flumioxazin was rapidly hydrolysed in all three buffered solutions and the half-lives were calculated to be 3.43 days, 18.9 - 23.9 hours and 14.0 - 15.1 minutes at pH 5, 7 and 9, respectively. Degradation proceeded via opening of the cyclo imide ring at all pH values to form 482-HA. Subsequent cleavage of the amide linkage to form major THPA (> 80 % after 30 days) and minor Δ^1-TPA was only observed at pH 7 and 5 (Table 22).

Table 22 Hydrolysis of [THP-^{14}C]-flumioxazin

	% of the applied radioactivity							
	pH 5			pH 7			pH 9	
Duration	1 d	4 d	30 d	1 d	7 d	30 d	30 min	30 d
Flumioxazin	75.5	41.0	-	32.1	16.2	3.5	29.2	-
482-HA	4.2	3.3	-	62.9	49.8	8.1	73.0	96.1
THPA	18.1	54.6	95.5	3.2	34.5	83.6	-	-
TPA	-	1.9	2.5	-	1.5	6.0	-	-

RMS Comments:

Tests on hydrolysis in buffered solutions were conducted under EPA-FIFRA 161-1. A review of this study indicates that it partially meets the current guideline OECD 111 (2004), deviations include:

- The hydrolysis test should be performed at pH value 4 instead of 5.
- Tiered testing at multiple temperatures was not conducted.

However, reconduct is unlikely to yield a significantly different result because the requirements of EPA guideline 161-1 are equivalent to OECD 111 and the study is scientifically valid.

Study 2: Hydrolytic degradation of [Ph-^{14}C]-flumioxazin

Guideline : EPA-FIFRA 161-1. Study performed in compliance with GLP. The study is valid.

Cross reference : IIA.7.2.1.1/02, Katagi *et al.*, 1990b.
This study was conducted as above, using [Ph-^{14}C]-flumioxazin (purity > 99 %).

Table 23: Hydrolysis of [Ph-^{14}C]-flumioxazin

	% of the applied radioactivity							
	pH 5			pH 7			pH 9	
Duration	1 d	4 d	30 d	1 d	7 d	30 d	30 min	30 d
Flumioxazin	81.2	44.9	-	40.7	19.8	5.8	34.3	-
482-HA	4.7	4.4	-	53.2	46.4	10.4	63.6	98.5
APF	13.2	46.3	86.8	3.8	33.0	80.0	-	-

The recovery of ^{14}C was 94.4 - 101.6 %. Radioactivity was fully recovered. Flumioxazin was rapidly hydrolysed in all three buffered solutions and the half-lives were calculated to be 4.91 - 5.20 days, 23.2 - 25.9 hours and 21.3 - 22.7 minutes at pH 5, 7 and 9, respectively. Degradation proceeded via opening of the cyclo imide ring at all pH values to form 482-HA. Subsequent cleavage of the amide linkage to form APF (> 80 % after 30 days) was only observed at pH 7 and 5. (Table 23)

RMS Comments:

Tests on hydrolysis in buffered solutions were conducted under EPA-FIFRA 161-1. A review of this study indicates that it partially meets the current guideline OECD 111 (2004), deviations include:

- The hydrolysis test should be performed at pH value 4 instead of 5.
- Tiered testing at multiple temperatures was not conducted.

However, reconduct is unlikely to yield a significantly different result because the requirements of EPA guideline 161-1 are equivalent to OECD 111 and the study is scientifically valid.

Metabolites

Study 3: Hydrolysis of hydrolytic metabolites

Guideline: In house method. Study not performed in compliance with GLP. The study is valid.
Cross reference: IIA.7.2.1.1/03, Katagi *et al.*, 1990c.
Hydrolysis kinetics of flumioxazin and its degradation product 482-HA were studied using their respective radiolabelled preparations, uniformly labelled at the phenyl moiety. [Ph-^{14}C]-flumioxazin or [Ph-^{14}C]-482-HA (purity > 99 %) were dissolved at 0.1 ppm in sterile buffers (pH value ranged from 2.5 to 9) using acetonitrile as a cosolvent (< 1 %). Solutions were kept at 25 ± 1 °C in the dark for 30 days. Analysis was performed by HPLC.
Flumioxazin showed an approximately constant rate of degradation under the acidic conditions at and below pH 5.0 (Table 24). The rate of degradation rapidly increased as pH increased above pH 5.0. Thus hydrolysis of flumioxazin proceeds predominantly through neutral and base catalyzed processes.
The degradation rate of 482-HA was higher under acidic condition and it decreased as pH increased especially above pH 4.5 (Table 24). Thus hydrolysis of 482-HA proceeds predominantly through an

acid catalyzed process. Half lives of 482-HA were calculated to be 2.35 hours, 10.7 days and 72 days at pH 5, 7 and 8 respectively.

Table 24: Influence of pH on hydrolysis rate of flumioxazin and its degradation product 482-HA

	Apparent hydrolysis rate (sec-1)	
pH	flumioxazin	482-HA
2.5	2.401×10^{-6}	5.222×10^{-4}
3.0	1.866×10^{-6}	4.858×10^{-4}
3.5	2.029×10^{-6}	4.387×10^{-4}
4.0	2.202×10^{-6}	3.692×10^{-4}
4.5	2.358×10^{-6}	2.437×10^{-4}
5.0	1.964×10^{-6}	8.205×10^{-5}
5.5	2.513×10^{-6}	3.295×10^{-5}
6.0	3.742×10^{-6}	1.503×10^{-5}
6.5	7.962×10^{-6}	3.825×10^{-6}
7.0	1.197×10^{-5}	7.490×10^{-7}
7.5	4.142×10^{-5}	3.215×10^{-7}
8.0	6.195×10^{-5}	1.119×10^{-7}
8.5	1.344×10^{-4}	-
9.0	6.599×10^{-4}	-

RMS Comments:
Study is considered as supportive.

Aqueous photolysis

Study 1: Photodegradation Study with [Ph-^{14}C]-flumioxazin

Guideline: EPA-FIFRA 161-2. Study performed in compliance with GLP. The study is valid.

Cross reference: IIA.7.2.1.2/01, Fathulla, 1995b.

A sterilised sodium acetate buffer solution at pH 5 was fortified with [Ph-^{14}C]-flumioxazin (purity > 99 %), at a nominal concentration of 0.1 µg/ml (acetonitrile < 1 % was used as cosolvent). Samples were kept at 25°C in the dark or exposed to xenon lamp (12h per day) for 30 days. Duplicate samples were removed from test conditions and analysed at 2, 5, 8, 24, 48, 96, 168, 264, 432 and 720 hours. Samples were flushed with nitrogen into a series of traps, which contained charcoal, ethylene glycol and 2-ethoxyethanol : ethanolamine (1:1). The radioactivity from the liquid traps was quantitated by LSC. The charcoal traps were combusted and counted by LSC. For radioactivity characterisation, samples were analysed by 2-D TLC and HPLC. The unknown metabolite corresponding to area 1 of TLC plates was tentatively identified by MS and NMR spectroscopy.
The mean total applied radioactivity recovedered from the samples renged from 93.8% to 100.0 % for the irradiated samples, and from 98.1% to 104.0% for the dark control samples. Radioactivity was fully recovered and no volatile compound was released. In the dark, DT_{50} of flumioxazin was 118 h and APF was the main degradation product. In the light, DT_{50} was 21 h. A metabolite (area 1) was rapidly formed (up to 74.6 %). MS and NMR data suggest that it would derive from

flumioxazin via opening of the phenyl ring and identified as 482-PHO. This compound progressively declined to 24 % whereas a number of unknown metabolites were formed (Table 25).

Table 25: Aqueous photolysis of [Ph-^{14}C]-flumioxazin – irradiated samples

Duration (days)	% of the applied radioactivity						
	Parent	Origin[a]	Area 1[b]	Area 2	APF	Area 4	Unknown
0	100.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2 hours	90.6	n.d.	8.9	n.d.	n.d.	n.d.	n.d.
5 hours	82.9	n.d.	16.6	n.d.	n.d.	n.d.	n.d.
8 hours	73.0	n.d.	24.2	n.d.	0.6	n.d.	n.d.
1	45.6	n.d.	50.2	n.d.	3.1	n.d.	n.d.
2	19.8	10.4	63.1	6.2	n.d.	n.d.	n.d.
4	5.7	11.8	74.6	7.0	n.d.	n.d.	n.d.
7	3.4	17.8	69.6	6.5	n.d.	n.d.	n.d.
11	2.5	30.2	57.1	6.4	n.d.	n.d.	n.d.
18	2.4	34.6	41.1	3.5	n.d.	8.5	5.1
25	4.7	37.1	29.7	2.2	n.d.	12.8	8.5
30	1.8	41.3	23.7	1.1	n.d.	16.8	9.0

n.d. = not detected
[a] consist of several unknown compounds
[b] identified as 482-PHO

Table B 26: Aqueous photolysis of [Ph-^{14}C]-flumioxazin – dark control

Duration (days)	% of the applied radioactivity						
	Parent	Origin*	Area 1	Area 2	APF	Area 4	Unknown
0	100.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2 hours	99.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5 hours	99.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8 hours	92.1	n.d.	n.d.	n.d.	6.0	n.d.	n.d.
1	85.0	n.d.	n.d.	n.d.	15.6	n.d.	n.d.
2	66.8	n.d.	n.d.	n.d.	33.1	n.d.	n.d.
4	55.7	n.d.	n.d.	n.d.	45.2	n.d.	n.d.
7	37.8	n.d.	n.d.	n.d.	61.7	n.d.	n.d.
11	16.6	n.d.	n.d.	n.d.	87.4	n.d.	n.d.
18	2.0	n.d.	n.d.	n.d.	97.5	n.d.	n.d.
25	n.d.	n.d.	n.d.	n.d.	99.8	n.d.	n.d.
30	n.d.	n.d.	n.d.	n.d.	101.1	n.d.	n.d.

n.d. = not detected

In conclusion, light slightly enhances degradation of flumioxazin in water at pH 5 and a different degradation pathway is involved. A new metabolite is rapidly formed via opening of the phenyl ring and further degraded to THPA and a lot of unknown degradation products.

RMS Comments:

Because of unknown compounds determined in this photolytic study, a new water/sediment study conducted under light/dark conditions has been submitted.

Study 2 (EU monograph, 1997): Photodegradation Study with [THP-^{14}C]-flumioxazin

Guideline: EPA-FIFRA 161-2. Study performed in compliance with GLP. The study is valid.
Cross reference: IIA.7.2.1.2/02, Fathulla, 1995a.

This study was conducted as described above, using [THP-^{14}C]-flumioxazin (purity > 99 %). Radioactivity was fully recovered and no volatile compound was released. In the dark, DT_{50} of flumioxazin was 96 h and THPA was the main degradation product. In the light, DT_{50} was 26 h. A metabolite (area 1) was rapidly formed (up to 54 % on day 2). This metabolite was the same as above. It progressively declined to 6.8 % whereas THPA (max. 23% AR) and a number of unknown metabolites were formed (Table 27).

Table 27: Aqueous photolysis of [THP-^{14}C]-flumioxazin – irradiated samples

Duration (days)	% of the applied radioactivity				
	Parent	Origin[a]	Area 1[b]	THPA	Area 3
0	100.0	n.d.	n.d.	n.d.	n.d.
2 hours	77.3	n.d.	25.2	n.d.	n.d.
5 hours	72.4	n.d.	26.6	3.7	n.d.
8 hours	69.2	n.d.	28.8	5.1	n.d.
1	54.5	n.d.	40.5	8.2	n.d.
2	24.1	13.7	54.1	12.1	n.d.
4	11.5	26.2	52.5	13.8	n.d.
7	10.0	33.6	40.7	20.2	n.d.
11	9.6	33.4	39.1	23.0	n.d.
17	10.9	30.9	21.2	20.5	19.4
21	13.7	40.4	14.7	15.2	19.3
26	11.6	26.3	16.0	18.4	28.5
30	8.8	35.2	6.8	13.4	35.1

n.d. = not detected
[a] consist of several unknown compounds
[b] identified as 482-PHO

Table 28: Aqueous photolysis of [THP-^{14}C]-flumioxazin – dark control

Duration (days)	% of the applied radioactivity				
	Parent	Origin	Area 1	THPA	Area 3
0	100.0	n.d.	n.d.	n.d.	n.d.
2 hours	100.9	n.d.	n.d.	n.d.	n.d.
5 hours	102.6	n.d.	n.d.	n.d.	n.d.
8 hours	100.7	n.d.	n.d.	3.2	n.d.
1	101.9	n.d.	n.d.	11.9	n.d.
2	102.6	n.d.	n.d.	24.1	n.d.
4	105.2	n.d.	n.d.	49.4	n.d.
7	106.1	n.d.	n.d.	76.3	n.d.
11	105.6	n.d.	n.d.	92.3	n.d.
17	106.4	n.d.	n.d.	102.9	n.d.
21	106.7	n.d.	n.d.	104.6	n.d.
26	105.8	n.d.	n.d.	105.0	n.d.
30	105.5	n.d.	n.d.	104.4	n.d.

n.d. = not detected

In conclusion, light slightly enhances degradation of flumioxazin in water at pH 5 and a different degradation pathway is involved. A new metabolite is rapidly formed via opening of the phenyl ring and further degraded to THPA and a lot of unknown degradation products.

RMS Comments:

Because of a lot of unknown compounds determined in this photolytic study, a new water/sediment study conducted under light/dark conditions has been submitted.

5.1.2 Biodegradation

Biodegradation of flumioxazin was examined in the following studies:

a) Study on ready biodegradability (Graham, R & Alderman, D., 2011, Report No.SBM-0086)

b) Water sediment study (Ridge M.A. (1998), report 1531/9-D2142)

c) Degradation of Flumioxazin in Illuminated Water-Sediment Systems (Shibata, A., Kodaka, R., Fujisawa, T & Katagi, T., 2011, Report No.: SBM-0088)

5.1.2.1 Biodegradation estimation

No estimation of biodegradation using QSAR sis available in the Renewal Assessment Report.

5.1.2.2 Screening tests

Report: Graham, R & Alderman, D. (2011)

Flumioxazin TG: Assessment of Ready Biodegradability by Measurement of CO2

Evolution

Sumitomo Chemical Co. Ltd Report No.SBM-0086

Guidelines: OECD No. 301 B Ready Biodegradability (Adopted 1981, Revised 1992)

GLP: Yes (laboratory certified by UK National Authority)

Test materials. Flumioxazin TG, purity 99.6 %

Validity: study is valid

Conclusion: Flumioxazin is not readily biodegradable under the conditions of the test.

Flumioxazin (15 mg C/L) was added to duplicate vessels containing mineral salts medium (3 L) inoculated with activated sludge (not previously knowingly exposed to test substance; 30 mg solids/L) and incubated in darkness for 28 days at 22°C.
Duplicate vessels containing inoculated mineral salts medium alone and duplicate vessels containing inoculated mineral salts medium plus the reference substance, sodium benzoate (15 mg C/L) were incubated in the same conditions. An additional mixture containing sodium benzoate and flumioxazin was established to assess the potential inhibitory effects of the test substance on the activity of the microbial inoculum.
During incubation the evolved carbon dioxide was measured at 2, 3, 6, 8, 10, 14, 17, 22, 27, 28 and 29 days. The amount of carbon dioxide absorbed by each barium hydroxide trap was calculated by titration of the remaining barium hydroxide with hydrochloric acid.
Biodegradation of sodium benzoate reached 78% after 14 days and 90% after 28 days. In the additional presence of flumioxazin, biodegradation of sodium benzoate reached 90% after 28 days, confirming that flumioxazin was not inhibitory to the activity of the microbial inoculum. These

results confirm that the inoculum was viable and the test result valid. Biodegradation of flumioxazin was 3% at the end of the test on Day 28.

RMS comments

Flumioxazin is not readily biodegradable under the conditions of the test. The study is acceptable. The results of the study are plausible.

5.1.2.3 Simulation tests

Study 1:

Ridge M.A. (1998), report 1531/9-D2142, GLP, SETAC guideline
Validity: study is valid.

Degradation of Flumioxazine (phenyl or THP labelled, purity > 97 %) was studied in 2 water sediment systems (clay loam, 8 % OC and sandy clay loam, 3.6 % OC) in accordance with the SETAC guideline (2.5 cm sediment + 6 cm water, 20° C). Flumioxazine was applied at a rate equivalent to 600 g/ha. Samples were collected 0, 7, 14, 29, 60 and 98 DAT and analysed by TLC, HPLC and LC-MS. The recovered radioactivity was in the range 88 - 103 % and was acceptable. After 98 d, mineralization was 27 - 31 % for the THP moiety and < 9 % for the phenyl moiety. Bound residue was 29 - 47 % for the THP moiety and 60 - 61 % for the phenyl moiety (associated with the humic fractions). Flumioxazine was found in sediment (max. 27 % after 7 d) and it rapidly disappeared in both water and sediment phases. For the whole systems, DT_{50} were < 1.85 d and DT_{90} were 25 - 69 d. Degradation occurred via hydrolysis to APF (max. 58 % in water after 7 d) and THPA (max. 63 % in water and 18 % in sediment after 7 d). These metabolites were further degraded to CO_2 and bound residue, and they did not persist (< 6 % after 98 d). A metabolite (SAT 482-HA-2) was found in small amounts in water (max. 5.6 % after 98 d) and sediment (max. 7 % after 60 d). Two unknown transient metabolites derived from the phenyl moiety were detected in significant amount in water on day 14. U@23.8 was detected at 21.8 % in one system and U@5.5 was detected at 17 % in the other system. These unknowns were clearly not persistent since they were < 3.4 % after 29 d. Despite further investigations, their chemical structure was not elucidated but results suggest that unknowns could derive from degradation of APF. Because of the very transient nature of the unknowns, the lack of identification is deemed to be acceptable as far as ecotoxicological risk assessment can be done.

Conclusions: Flumioxazine is rapidly degraded in water sediment systems with DT_{50} < 1.85 d and DT_{90} 25 - 69 d (whole system), and temporarily found in sediment (max. 27 % after 7 d). Degradation occurs via hydrolysis to APF (max. 58 % in water after 7 d) and THPA (max. 63 % in water and 18 % in sediment after 7 d) which are the major degradation products. These metabolites are further degraded to CO_2 (max. 31 % THP and < 9 % phenyl after 98 d) and bound residue (max. 47 % THP and 61 % phenyl after 98 d). Two unknown transient metabolites derived from the phenyl moiety (and probably from degradation of APF) are detected in significant amount in water (17 and 22 % on day 14). Identification is not required as far as ecotoxicological risk assessment can be done.

Table 29: Degradation of THP-^{14}C flumioxazine in water sediment systems

	% of applied RA						
DAT	Flumioxazine	THPA	U@5.5	Unknowns	Bound	CO_2	Recov.

	Sed.	Water	Sed.	Water	Sed.	Water	Sed.	Water	(NE)		
Millstream (clay loam pH 7.4 in water and 0.01 M $CaCl_2$, water pH 7.8)											
0	10.3	77.7	1.1	5.6	-	-	-	-	0.1	-	98.3
7	11.3	2.0	18.6	47.2	1.2	-	-	10.2	4.4	0.2	97.0
14	16.0	1.2	12.9	49.2	2.0	-	-	0.8	9.0	0.6	97.5
29	14.6	0.7	8.7	32.4	1.5	3.1	-	1.9	19.9	2.6	93.2
60	8.9	0.4	3.6	18.9	1.2	2.2	-	3.3	28.7	10.3	88.8
98	4.4	0.6	1.3	5.7	1.5	4.6	1.9	0.8	28.9	26.7	88.4
Emperor Lake (sandy clay loam pH 6.4 in water and 5.4 in 0.01 M $CaCl_2$, water pH 6.3)											
0	7.5	75.5	-	5.1	-	-	-	-	0.1	-	92.4
7	10.0	2.0	6.5	62.8	0.3	-	0.5	6.5	5.8	0.2	97.4
14	4.0	2.3	13.5	60.4	1.3	2.1	-	0.9	9.0	0.2	96.9
29	12.9	1.6	5.4	50.0	1.0		0.3	0.7	18.7	0.5	96.9
60	8.9	1.2	0.3	2.4	2.7	1.7	0.8	2.0	58.0	6.8	91.7
98	4.0	-	-	0.7	1.9	-	-	-	47.0	30.9	89.6

IMOXA < 2.7 %, 482-HA < 0.5 %, Sat-482-HA-2 < 4.6 % in each phase

Table 30: Degradation of phenyl-^{14}C flumioxazine in water sediment systems

DAT	% of applied RA												
	Flumioxazine		APF		U@5.5		U@23.8		Unknowns		NE	CO_2	Rec.
	Sed.	Water	Sed.	Water	Sed.	Water	Sed.	Water	Sed.	Water			
Millstream													
0	10.7	62.9		24.2	-	-	-	-	-	-	-	-	103.1
7	26.8	2.2	5.0	42.0	-	1.7	-	-	-	9.7	3.8	-	97.6
14	17.4	5.4	4.2	-	3.2	5.6	-	21.8	2.3	21.0	8.0	0.1	97.5
29	22.0	1.7	0.9	16.1	0.9	4.7	1.7	3.4	0.5	5.2	27.8	0.4	96.7
60	9.8	0.3	3.2	12.7	-	-	0.6	-	1.7	3.3	47.7	1.5	96.8
98	5.1	0.4	2.4	4.1	-	-	0.7	-	1.1	1.1	61.3	1.7	96.4
Emperor Lake													
0	12.0	73.4	-	1.7	-	-	-	-	-	5.4	0.1	-	98.1
7	9.6	2.4	-	57.7	-	5.6	-	-	11.3	5.1	5.0	-	98.1
14	9.8	3.3	4.9	2.9	1.0	17.2	-	5.0	5.5	26.0	9.8	0.1	97.3
29	18.2	14.8	0.4	2.8	-	2.6	2.2	6.8	2.3	13.3	22.7	0.2	96.6
60	10.5	1.1	-	1.2	-	1.4	3.4	1.3	2.0	4.4	53.8	3.3	94.7
98	5.3	-	-	0.5	-	0.8	2.4	-	3.4	3.1	60.0	9.2	95.2

IMOXA < 4.1 %, 482-HA < 0.6 %, 482-CA < 1.9 %, SAT-482-HA-2 < 7 % in each phase, unknowns < 7.1 % each

RMS Comments:
Study is accepted. Recalculation of kinetics data according to the current FOCUS guidance has been submitted. Because two unknown transient metabolites were detected, a new water/sediment study has been performed.

Study 2:

Report: Jarvis, T. & Mamouni, A (2011b)
Recalculation of Flumioxazin sediment water kinetics according to FOCUS (2006) Guidance
Sumitomo Chemical Co. Ltd Report No.: SBM-0084
Guidelines: FOCUS (2006)
GLP: No, not applicable to simulation modeling
Validity: study is valid
Conclusion: The whole system DT50 values were calculated from DT90 (DT90/3.32) with the geometric mean value of 21.6 days.

The rates of dissipation/degradation of flumioxazin from the existing water sediment study previously evaluated (Ridge, 1998), were recalculated according to current FOCUS guidance using Kingui 1.1 and ModelMaker 4.
Data for the THP- and phenyl-labelled flumioxazin were treated as true replicates and hence one set of $DT_{50/90}$ values was determined for the Millstream system and one for the Emperor Lake system. Following the FOCUS kinetics (2006) recommendations, the optimised parameters and associated statistics for SFO, FOMC and DFOP kinetics for the whole system and the water phase only (i.e. P-I approach), are shown in Table 31. Although SFO kinetics showed acceptable fits for the water phase of both systems, in all cases (whole system or water phase only) the best fit was shown with DFOP kinetics (Figures 3 and 6 for whole systems).
P-II approaches were undertaken using ModelMaker 4. The results in Table 32 show that the χ^2 error (%) value for the water phase is acceptable and the visual fit is good (Figure 13 and 14), but the t-test does not show significance. In contrast, the χ^2 error (%) value for the sediment phase is very large and the visual fit is poor (Figure 13 and 14). This is partly due to the fact that most of the flumioxazin ultimately reaching the sediment was already present at the day 0 timepoint. The degradation rate in both sediments is predicted to be negative which invalidates the fit in those systems. Overall, the full acceptance criteria for the use of P-II results are not considered to have been met.

Table 31: Summary of the results of the kinetic determinations for flumioxazin from water sediment studies (P-I approach)

Parameter	Millstream system	Emperor Lake system	Millstream water phase	Emperor Lake water phase
Model	SFO	SFO	SFO	SFO
χ^2 error (%)	30.5	27.9	8.8	6.7
k (day^{-1}) *	0.1308 (0.0012)	0.2493 (7.4×10^{-4})	0.4877 (0.0073)	0.4912 (1.8×10^{-4})
DT_{50} (day)	5.3	2.8	1.4	1.4
DT_{90} (day)	17.6	9.2	4.7	4.7
Model	FOMC	FOMC	FOMC	FOMC
χ^2 error (%)	10.0	9.7	4.6	2.7
α*	0.3641	0.1721	0.5918	0.5848

	(0.0263)	(0.0797)	(0.2840)	(8.9×10^{-4})
β*	0.2459 (0.3093)	1.0×10^{-4} (0.4511)	0.0319 (0.4614)	0.0290 (0.2037)
DT_{50} (day)	1.4	0.006	0.07	0.1
DT_{90} (day)	137	65.2	1.5	1.5
Model	DFOP	DFOP	DFOP	DFOP
χ^2 error (%)	5.6	9.2	4.2	1.9
k1*	3.3641 (0.5)	1.9992 (0.4999)	2.1179 (0.5)	2.2762 (0.4999)
k2*	0.0141 (0.0230)	0.0065 (0.0831)	0.0268 (0.3636)	0.0148 (0.1450)
g*	0.6938 (2.6×10^{-6})	0.8482 (1.4×10^{-8})	0.9531 (2.3×10^{-6})	0.9618 (6.6×10^{-9})
DT_{50} (day)	0.4	0.4	0.4	0.3
DT_{90} (day)	79.5	64.5	1.3	1.2

*P value from the t-test is given in brackets.

Table 32: P-II SFO degradation rates of flumioxazin in the water and sediment phases of water sediment systems

compartment	K	Standard error value	χ^2 error (%)	T- test (p value)	$DegT_{50}$ (day)
Water					
Millstream	0.470	0.861	4.6	0.296	1.5
Emperor Lake	0.540	0.514	3.9	0.154	1.3
Sediment					
Millstream	-0.034	0.132	27.9	-	-
Emperor Lake	-0.080	0.131	42.3	-	-

Figure 1: Millstream system – SFO kinetics

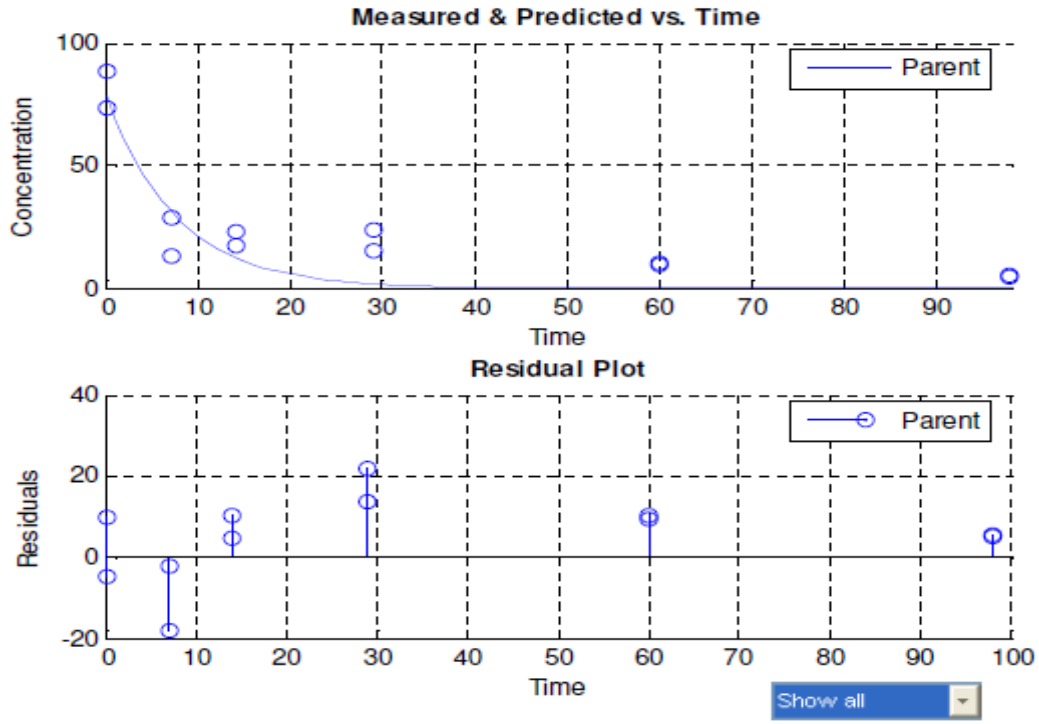


Figure 2: Millstream system – FOMC kinetics

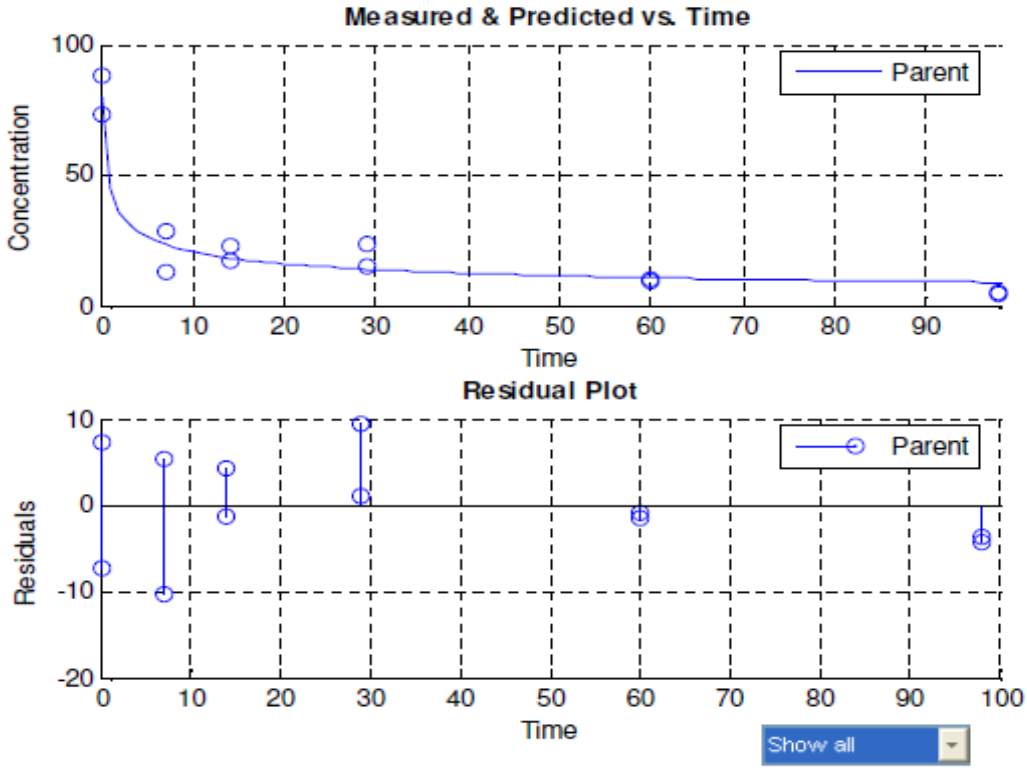


Figure 3: Millstream system – DFOP kinetics

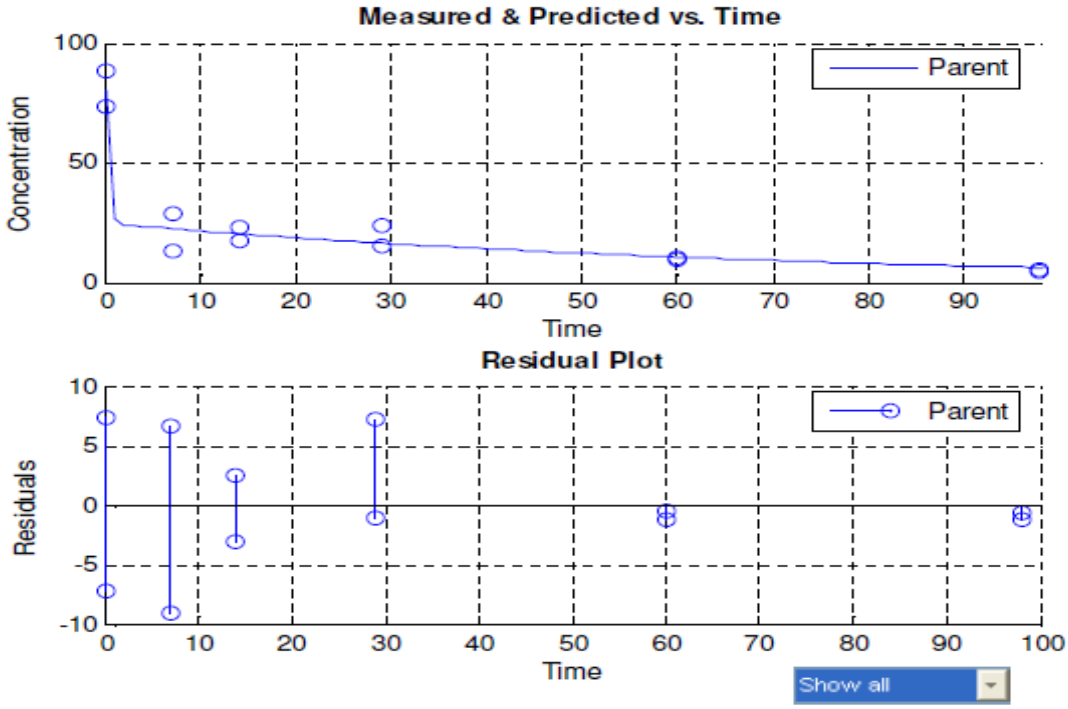


Figure 4: Emperor Lake system – SFO kinetics

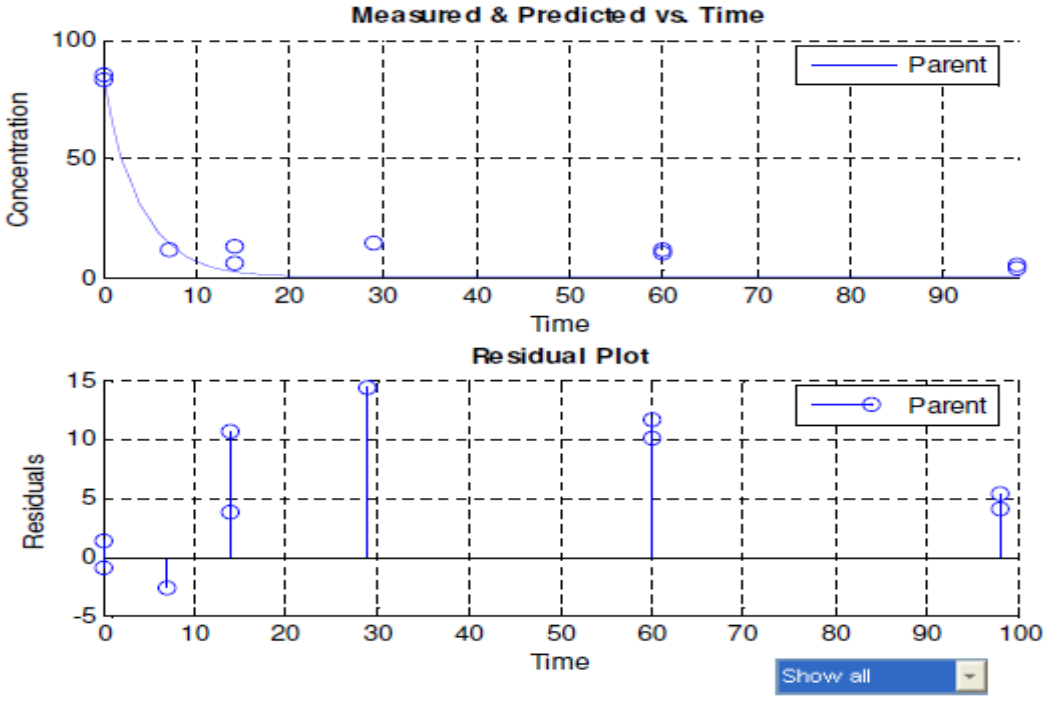


Figure 5: Emperor Lake system – FOMC kinetics

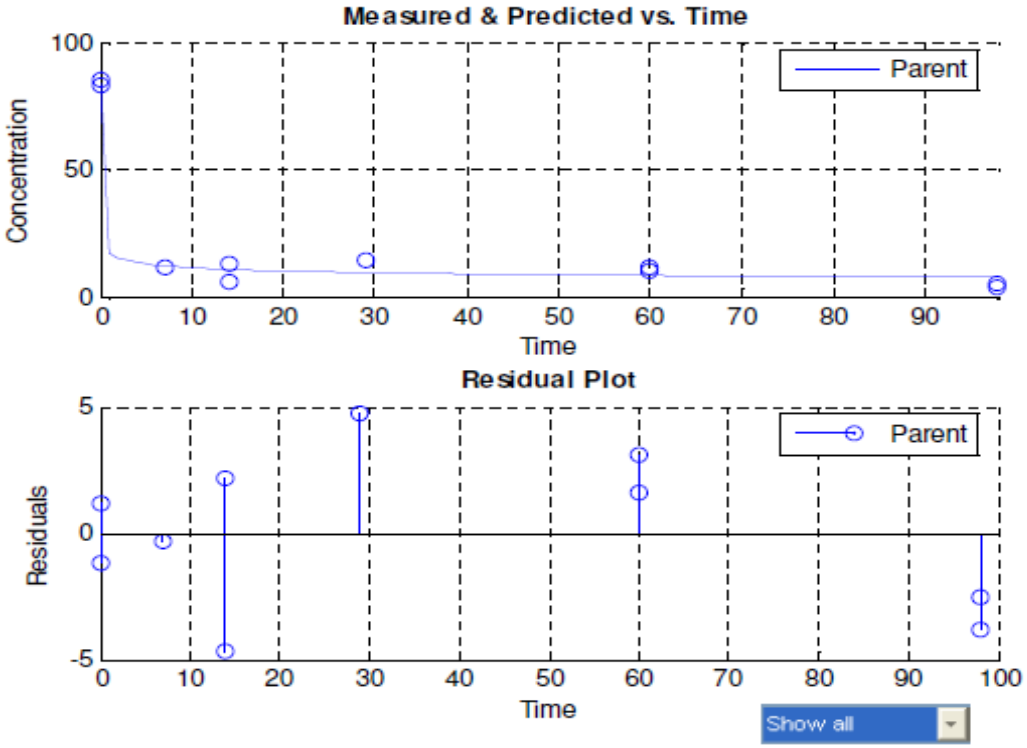


Figure 6: Emperor Lake system – DFOP kinetics

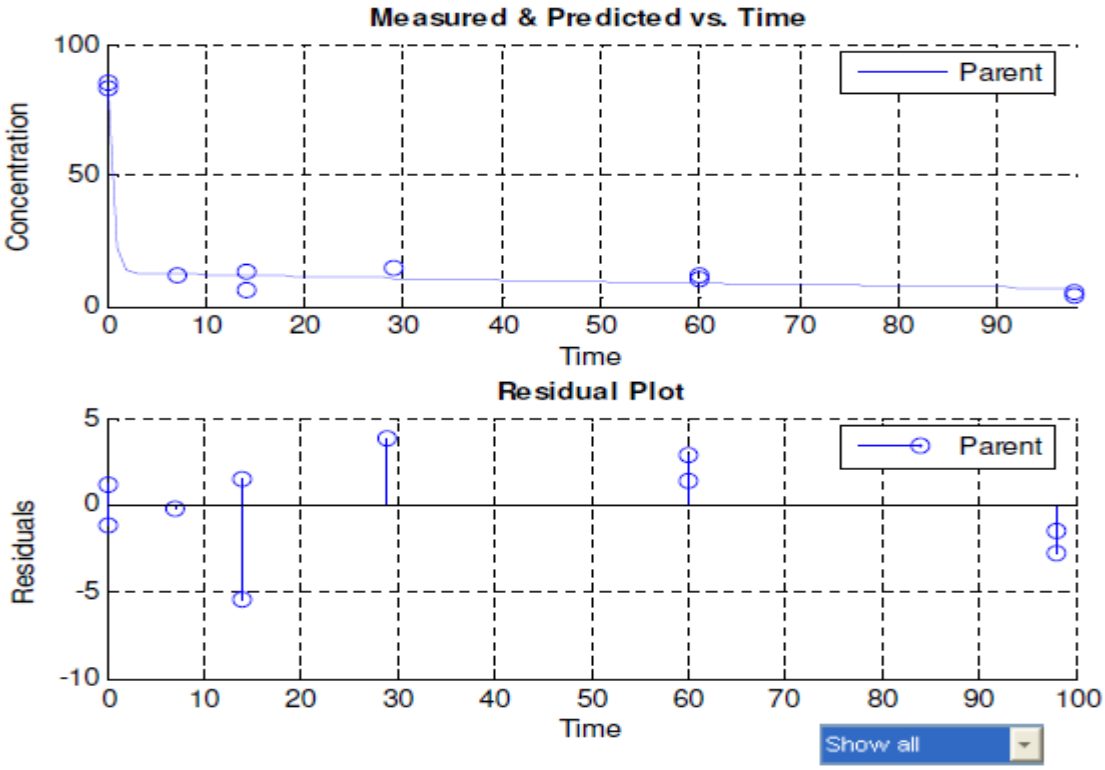


Figure 7: Millstream water – SFO kinetics

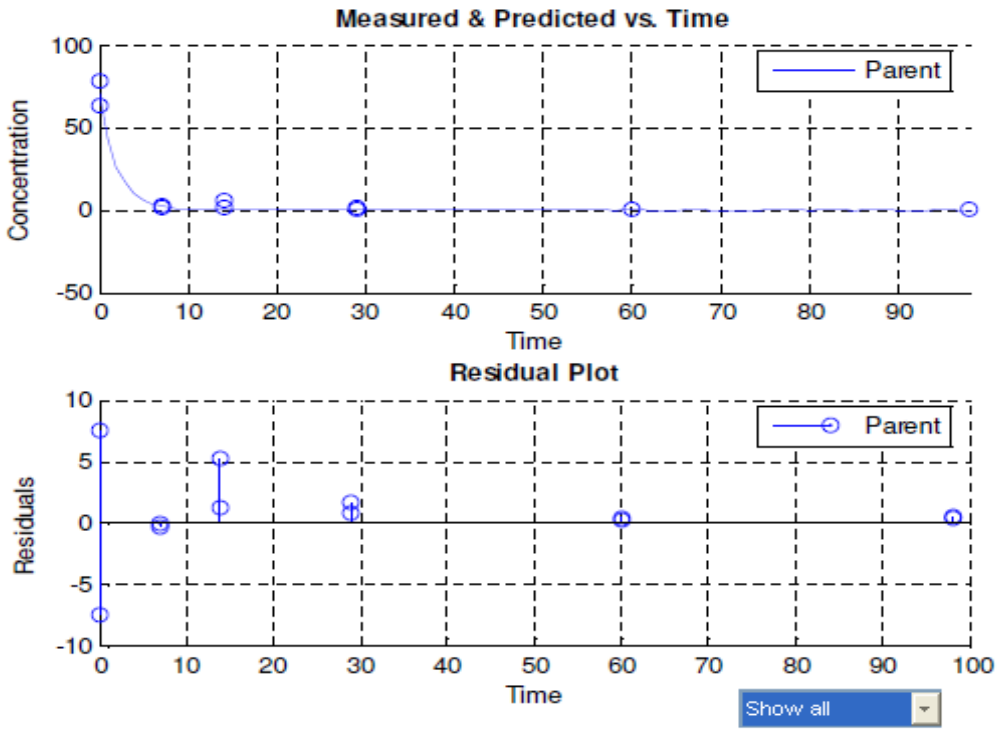


Figure 8: Millstream water – FOMC kinetics

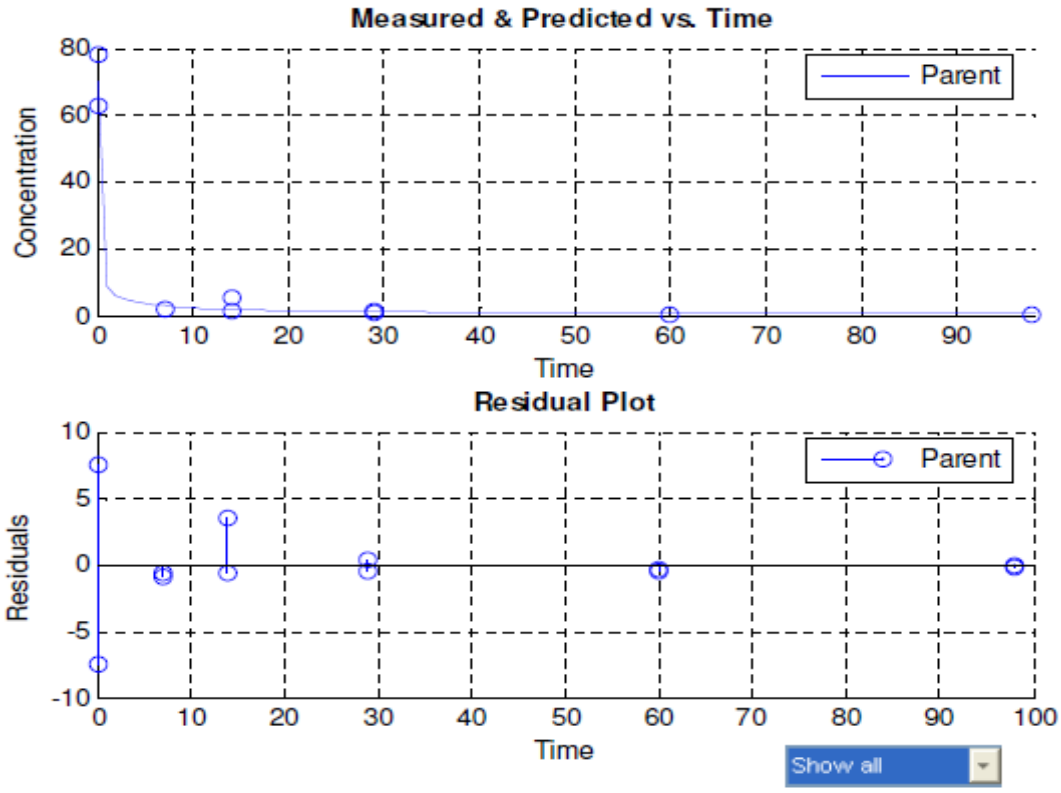


Figure 9: Millstream water – DFOP kinetics

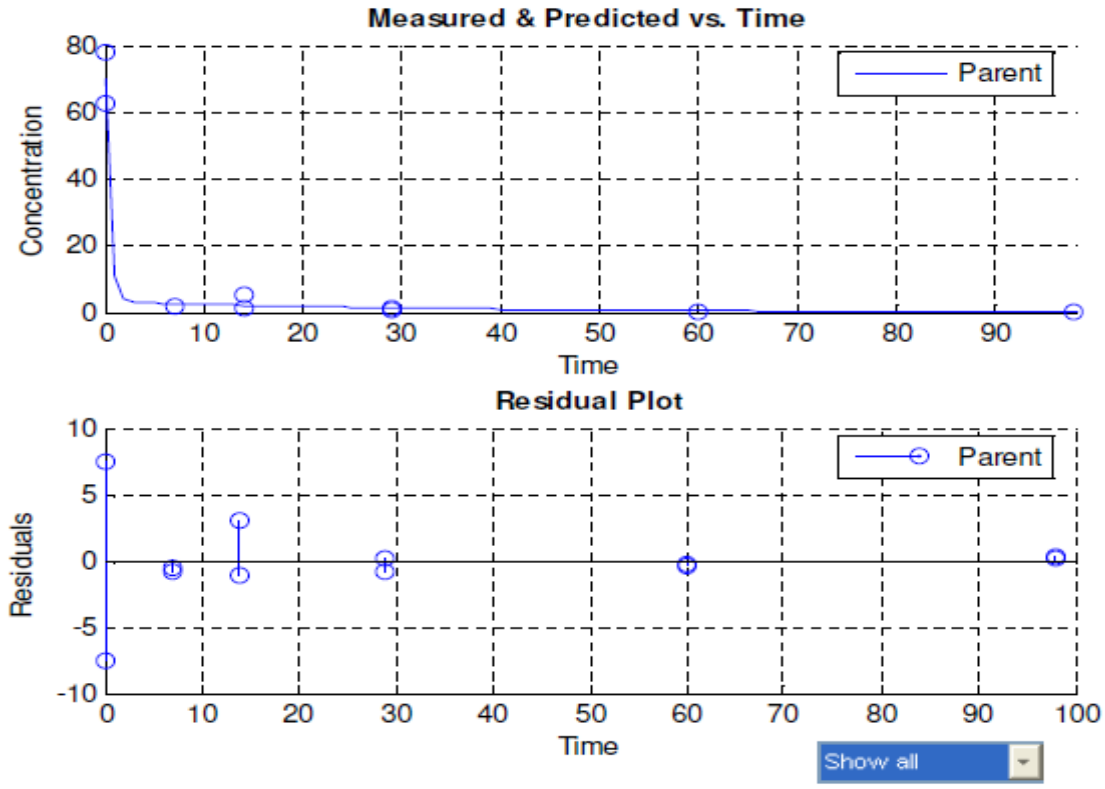


Figure 10: Emperor Lake water – SFO kinetics

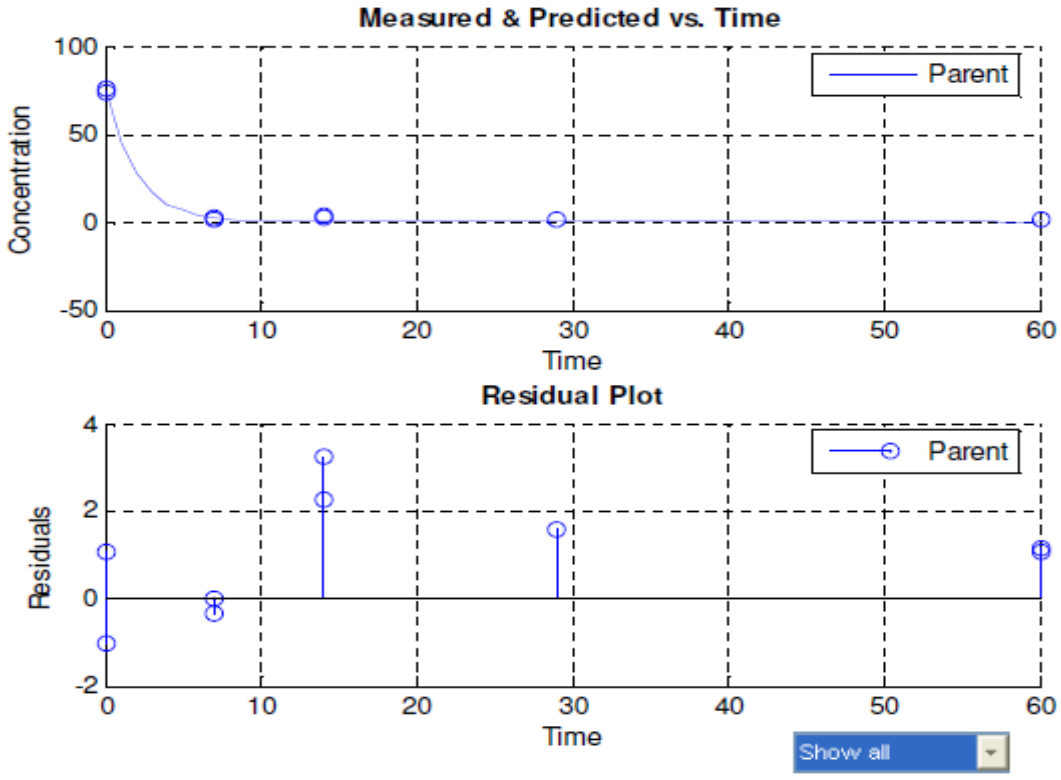


Figure 11: Emperor Lake water – FOMC kinetics

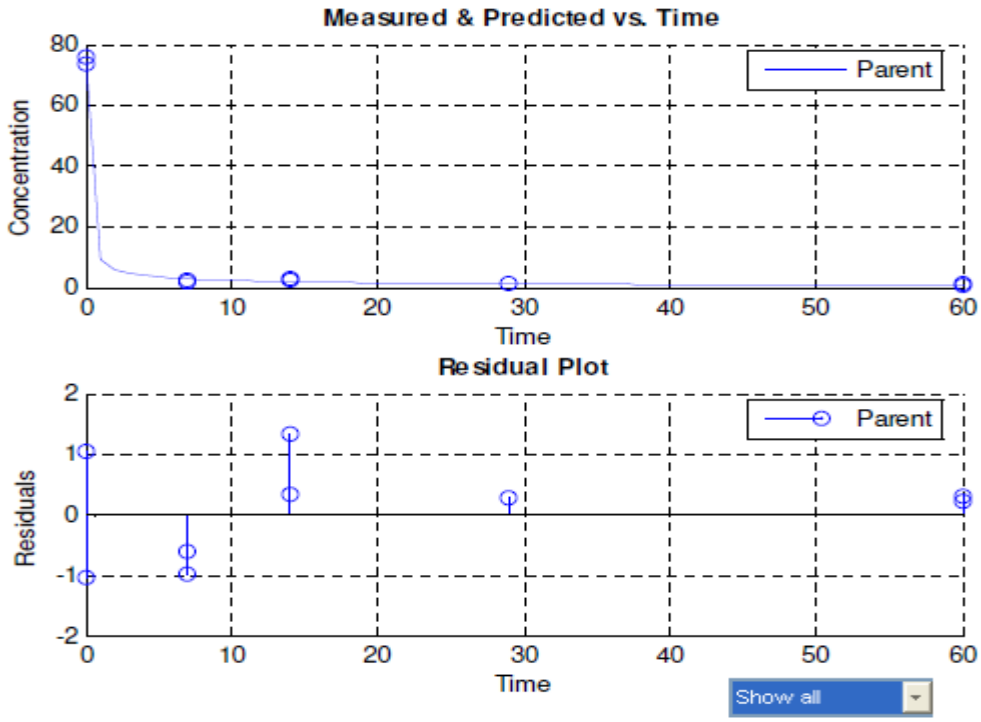


Figure 12: Emperor Lake water – DFOP kinetics

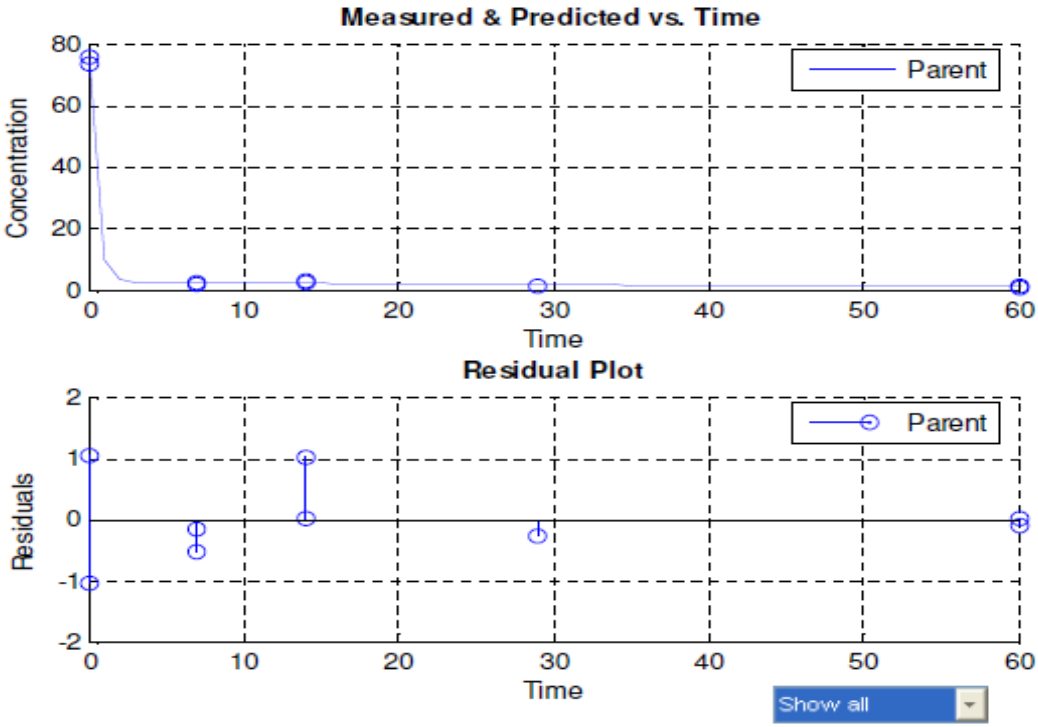


Figure 13: P-II Millstream sediment and water phases – SFO kinetics

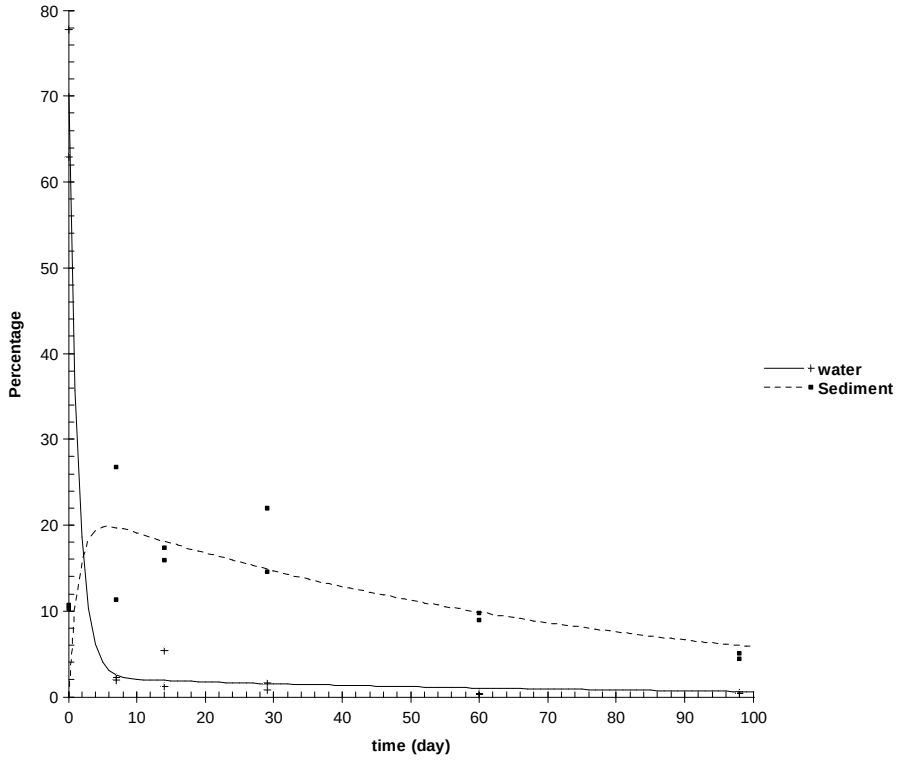
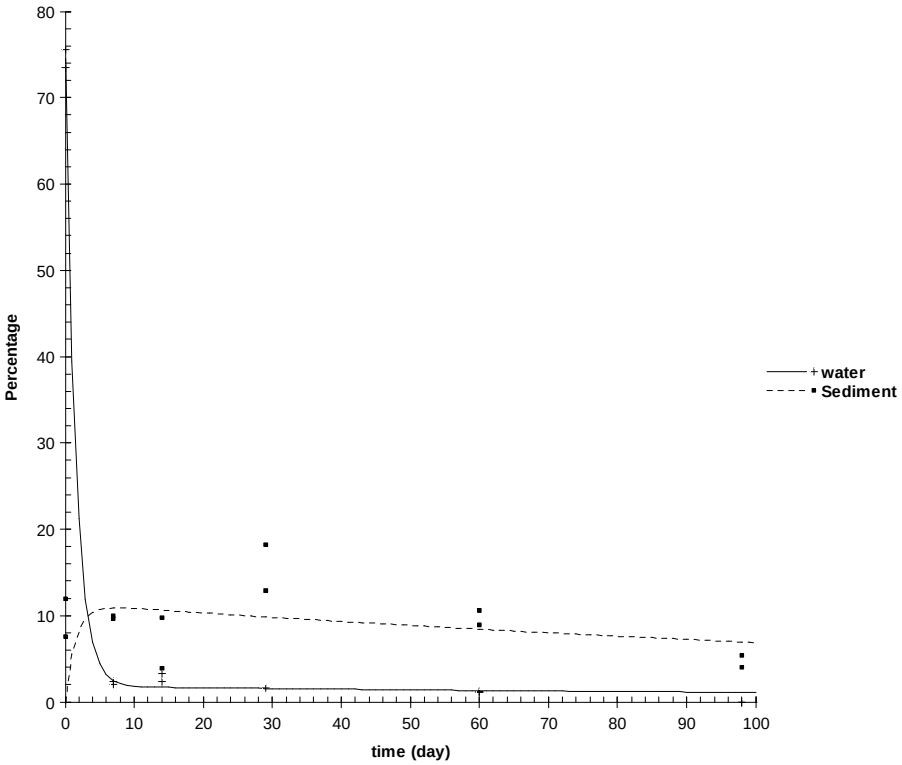


Figure 14: P-II Emperor Lake sediment and water phases – SFO kinetics



Conclusion (proposed by the Notifier):

As the degradation in water is clearly faster than in sediment, the P-I value for the whole system has been used to derive the endpoint for the slower phase. The best fit kinetics in the whole system is DFOP and the slow phase degradation rates are 0.0141 d^{-1} and 0.0065 d^{-1} in Millstream and Emperor Lake, respectively. This results in $DegT_{50}$ values of 49.1 and 107 days, respectively and a geometric mean DT_{50} value for modelling of 72 days.
The full P-II acceptance criteria were not met. However, flumioxazin is known to rapidly hydrolyse at neutral pH (DT_{50} = 19-26 hr) and hence this additional information can be utilised to help determine appropriate simulation endpoint values. SFO kinetics showed acceptable visual fits, as judged by the χ^2 error (%) value for both the dissipation from the water phase (P-I) and the degradation from the water (P-II), and there was no systematic bias in the residuals. Therefore, taking into account that hydrolysis is a known major pathway of degradation, the appropriate $DegT_{50}$ for use in modelling is considered to be an SFO value of 1.5 days. This is the $DegT_{50}$ calculated for Millstream and is the most conservative value of the SFO data for dissipation or degradation in water in either system.

RMS Comments:

Recalculation of kinetics values followed FOCUS Degradation Kinetics guidence. Appropriate DegT50 for use in modelling is the best fit kinetics in the whole system DFOP, DT50 values were calculated from DT90 (DT90/3.32) with the geometric mean value of 21.6 days. As no reliable DegT50 values have been obtained for water and sediment, the geomean whole system DT_{50} value will be used for the water phase and the default value of 1000 days was used for the sediment phase, instead of 72 days (sediment) and 1.5 days (water) proposed by the Notifier. Because the Koc value of flumioxazin is between 100 – 2000 mL/g the second approach of the default for the water phase and the whole system value for the sediment was modelled.

Study 4:

Report: Shibata, A., Kodaka, R., Fujisawa, T & Katagi, T. (2011)
Degradation of Flumioxazin in Illuminated Water-Sediment Systems
Sumitomo Chemical Co., Ltd. Report No.: SBM-0088

Guidelines: OECD 308 was used as a basis as there is no fixed study design currently available for illuminated conditions
GLP: No, published paper
Validity: study is valid

Test Materials: [Phenyl-^{14}C]Flumioxazin
[THP-^{14}C]Flumioxazin
Purity: [Phenyl-^{14}C]Flumioxazin: radiochemical purity >97%
[THP-^{14}C]Flumioxazin: radiochemical purity >97%
Water/sediment: Samples of sediment and associated water were collected from two sites and stored at 4ºC until use. Characteristics are shown in Table B.8.4.4-6.

Table 33: Characteristics of the sediment and water used

Characterisation	Kasai pond, Japan	Calwich Abbey Lake, UK
Sediment		
Particle size distribution[1]		
% Sand	14	27
% Silt	56	68
% Clay	30	5
Classification[1]	Silty clay loam	Silt loam
Organic carbon	1.9	4.9
pH (H_2O)	6.8	7.9
Overlying water		
Suspended solids (mg/l)	42.9	16.2
pH	6.9	7.9

Two systems (Calwich Abbey Lake and Kasai Pond) were set up containing natural sediment (2 cm) and associated water (6 cm) and suitable traps (ethylene glycol, alkaline) for collecting volatile compounds. The system was gently agitated and the flasks were allowed to equilibrate for at least 14 days at 20°C. Vessels were illuminated (300-400 nm; 0.672 MJ/m^2/day) for 8 hr per day and suitable dark controls were also provided. [Phenyl-^{14}C]Flumioxazin or [THP-^{14}C]flumioxazin (5.47 µg, equivalent to 600 g a.s./ha) dissolved in acetonitrile (20 µl) was added to the water surface. The flasks were then incubated in the illuminated conditions, or as dark controls, at 20°C for up to 30 days. Similar incubations were undertaken using the water only.

Duplicate units were taken at 0, 1 (THP label only), 3, 7, 14, and 30 days for the water sediment systems, or at 0, 0.04, 0.17 (both Calwich Abbey Lake only), 0.25, 0.5 (Calwich Abbey Lake only), 0.67 (Kasai Pond only), 1 3, 7, 14 and 30 days for the water only systems.
Water was separated from sediment. Overlying water was directly analysed by HPLC. Sediment samples were extracted with acetone: water (5:1 v/v) and then acetone: 0.1 M HCl (9:1 v/v). These sediment extracts were concentrated and analysed by HPLC. Metabolites were identified by co-chromatography with authentic standards. Unextractable soil residues were determined by combustion/LSC.

The mean results from the duplicate applications are shown in Tables 34 to 38. 482-HA, THPA and APF were present in both illuminated and dark systems, whereas 482-PHO and PHO-HA were only found in illuminated systems, confirming that they are photolysis metabolites. Both compounds exceeded 10% in at least one of the two systems. The uknown fractions in "others" exceeded neither 10%AR nor >5% AR in at least two sequential measurements in the water-sediment systems. In the natural water system there were several unknown fractions in "others", but none exceeded 9.1% AR (Tables 37 to 38).

The following compound codes are used:
482-HA= *N*-[7-fluoro-3-oxo-4-(2-propynyl)-2*H*-1,4-benzoxazin-6-yl]-3,4,5,6-tetrahydrophthalamic acid
THPA = 3,4,5,6-tetrahydrophthalic acid
APF = 6-amino-7-fluoro-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one
482-PHO = *N*-(2-propynyl)-4-[4-carboxy-3-fluoro-2-(3,4,5,6-tetrahydrophthalimido)-2-butenylidene]azetidine-2-one

PHO-HA = *N*-(2-propynyl)-4-[4-carboxy-3-fluoro-2-(2-carboxy-1-cyclohexencarbonylamino)-2-butenylidene]azetidine-2-one

Table 34: Distribution of radioactivity following incubation of [THP-^{14}C]Flumioxazin in Kasai Pond water sediment system

Compounds	Illumination						Darkness					
	0 day	1 day	3 day	7 day	14 day	30 day	0 day	1 day	3 day	7 day	14 day	30 day
Volatiles (CO_2)	na	nd	0.3	4.2	8.3	24.0	na	nd	0.1	1.1	4.2	8.3
Water phase												
Flumioxazin	87.1	54.5	23.2	3.4	1.1	nd	94.8	53.3	11.9	9.9	3.5	0.5
482-HA	3.3	14.3	17.2	5.3	nd	nd	3.8	21.3	55.9	27.7	26.5	15.5
THPA	nd	5.6	10.9	9.4	5.4	nd	nd	3.9	6.4	7.5	10.3	5.7
482-PHO	nd	2.6	5.4	10.2	2.6	nd	nd	nd	nd	nd	nd	nd
PHO-HA	nd	0.5	3.3	9.8	2.7	1.7	nd	nd	nd	nd	nd	nd
Other unknowns	nd	1.1	0.7	2.6	0.8	nd	nd	0.4	nd	1.3	3.2	2.4
Sediment phase												
Flumioxazin	7.1	15.5	18.7	9.8	5.0	5.7	na	14.4	12.9	16.4	8.3	6.7
482-HA	nd	2.0	4.4	4.0	5.9	1.8	na	1.0	7.3	7.6	15.4	6.5
THPA	nd	nd	0.6	0.9	nd	1.2	na	nd	nd	nd	nd	2.5
482-PHO	nd	nd	0.7	2.0	2.0	nd	na	nd	nd	nd	nd	nd
PHO-HA	nd	nd	nd	0.3	0.9	0.2	na	nd	nd	nd	nd	nd
Other unknowns	0.3	nd	0.4	4.0	3.7	1.9	na	1.1	nd	2.3	nd	1.2
Bound residues	0.6	4.0	13.8	27.0	53.6	48.0	0.1	2.0	4.1	20.3	25.0	36.9
TOTAL	98.3	100.6	99.9	92.9	92.0	90.6	100.0	98.1	99.9	94.1	96.4	86.2

nd = not detected, na = not analysed

Table 35: Distribution of radioactivity following incubation of [phenyl-^{14}C] Flumioxazin in Kasai Pond water sediment system

Compounds	Illumination					Darkness				
	0 day	3 day	7 day	14 day	30 day	0 day	3 day	7 day	14 day	30 day
Volatiles (CO_2)	na	1.7	7.4	8.8	24.5	na	0.3	0.7	1.1	2.5
Water phase										
Flumioxazin	91.6	9.1	2.6	nd	nd	91.6	30.6	10.5	2.7	nd
482-HA	2.3	27.9	6.1	nd	nd	2.3	17.3	23.7	33.9	3.7
APF	nd	1.2	0.8	nd	nd	nd	4.9	2.5	nd	0.7
482-PHO	nd	9.4	7.2	1.7	nd	nd	nd	nd	nd	nd
PHO-HA	nd	11.1	17.4	6.7	nd	nd	nd	nd	nd	nd
Other unknowns	1.4	5.4	8.9	21.9	3.8	1.4	3.6	1.4	1.0	nd
Sediment phase										
Flumioxazin	2.0	13.4	8.4	4.2	3.6	2.0	8.5	16.9	13.5	16.9
482-HA	nd	4.9	3.8	2.9	2.1	nd	18.6	10.2	10.5	5.6
APF	nd	0.9	2.5	2.0	nd	nd	1.1	2.6	3.3	3.1
482-PHO	nd	1.2	1.4	nd	0.7	nd	nd	nd	nd	nd
PHO-HA	nd	nd	0.3	nd	0.2	nd	nd	nd	nd	nd
Other unknowns	nd	1.9	4.1	5.7	6.6	nd	1.8	3.3	3.6	2.8
Bound residues	0.1	11.8	24.7	35.6	55.3	0.1	14.4	26.6	30.3	61.4
TOTAL	97.4	99.8	95.6	89.6	96.9	97.4	101.0	98.3	99.9	96.6

nd = not detected, na = not analysed

Table 36: Distribution of radioactivity following incubation of [THP-^{14}C]Flumioxazin in Calwich Abbey Lake water sediment system

Compounds	Illumination						Darkness					
	0 day	1 day	3 day	7 day	14 day	30 day	0 day	1 day	3 day	7 day	14 day	30 day
Volatiles (CO_2)	na	nd	0.4	2.1	7.2	7.5	na	nd	0.1	0.3	1.0	7.8
Water phase												
Flumioxazin	86.0	1.1	nd	nd	nd	nd	86.0	0.9	1.2	1.8	nd	nd
482-HA	6.4	60.9	30.1	6.4	1.9	0.8	6.4	75.8	76.1	64.2	48.1	33.9
THPA	nd	2.9	15.9	28.8	23.0	7.9	nd	nd	0.9	3.0	3.4	nd
482-PHO	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PHO-HA	nd	9.9	11.6	7.0	1.7	nd	nd	nd	nd	nd	nd	nd

Other unknowns	nd	4.5	13.5	14.9	12.8	8.7	nd	4.4	4.2	3.1	1.5	7.4
Sediment phase												
Flumioxazin	2.2	2.3	2.1	1.8	1.2	nd	2.2	1.8	0.8	3.1	7.3	6.5
482-HA	nd	9.9	12.3	5.8	nd	2.4	nd	11.2	12.8	13.9	18.7	20.4
THPA	nd	nd	0.5	2.0	6.6	4.5	nd	nd	nd	0.2	1.0	0.3
482-PHO	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
PHO-HA	nd	1.1	1.8	1.7	6.2	nd	nd	nd	nd	nd	nd	nd
Other unknowns	nd	1.4	3.1	5.1	2.9	8.7	nd	0.9	1.4	2.3	3.1	3.5
Bound residues	0.1	1.7	6.2	16.9	26.2	40.4	0.1	0.7	1.1	3.0	10.0	15.8
TOTAL	97.7	95.6	97.5	92.5	89.8	80.9	97.6	95.8	98.5	95.0	94.1	95.6

nd = not detected, na = not analysed

Table 37: Distribution of radioactivity following incubation of [THP-^{14}C]Flumioxazin in Kasai Pond natural water system

Compounds	0 day	0.25 day	0.67 day	1 day	3 day	7 day	14 day	30 day
Illuminated								
Volatiles (CO_2)	na	na	na	nd	0.1	0.8	1.9	14.8
Flumioxazin	94.8	45.6	21.4	6.6	1.8	nd	nd	nd
482-HA	6.1	44.7	63.8	80.3	40.9	7.9	nd	nd
THPA	nd	1.2	2.4	1.3	6.9	24.2	37.6	59.1
482-PHO	nd	0.5	0.4	1.7	7.4	11.0	11.0	3.2
PHO-HA	nd	4.2	7.6	10.0	35.3	50.8	37.6	15.2
Other unknowns	nd	nd	0.1	0.6	6.9	5.4	9.2	8.3
TOTAL	101.3	96.5	98.1	100.8	99.5	100.4	97.6	101.3
Darkness								
Volatiles (CO_2)	na	na	na	nd	nd	nd	0.1	0.1
Flumioxazin	94.8	59.9	29.6	7.0	2.1	1.4	2.5	0.9
482-HA	6.1	36.6	68.1	89.9	93.9	89.0	76.1	61.0
THPA	nd	nd	nd	1.3	4.3	10.2	21.6	35.9
482-PHO	nd	nd	nd	nd	nd	nd	nd	nd
PHO-HA	nd	nd	nd	nd	nd	nd	nd	nd
Other unknowns	nd	0.5	0.3	1.7	1.2	1.2	1.9	2.2
TOTAL	101.3	97.3	98.4	100.3	102.0	102.1	102.3	100.4

nd = not detected, na = not analysed

Table 38: Distribution of radioactivity following incubation of [THP-^{14}C]Flumioxazin in Calwich Abbey Lake natural water system

Compounds	0 day	0.04 day	0.17 day	0.25 day	0.5 day	1 day	3 day	7 day	14 day	30 day
Illuminated										
Volatiles (CO_2)	na	na	na	na	na	nd	0.1	0.4	2.9	9.0
Flumioxazin	92.7	62.3	15.7	6.2	nd	nd	nd	nd	nd	nd
482-HA	4.8	38.6	77.2	82.4	73.7	67.3	44.7	4.5	nd	nd
THPA	nd	0.0	0.7	1.0	3.1	3.3	11.4	24.1	57.4	46.9
482-PHO	nd	nd	nd	nd	Nd-	nd	nd	2.1	nd	0.8
PHO-HA	nd	0.0	5.9	11.5	20.5	14.8	33.9	48.5	18.7	nd
Other unknowns	0.4	0.4	1.0	1.8	1.8	10.6	7.1	15.3	15.9	34.9
TOTAL	98.3	101.6	101.7	103.2	99.5	96.7	97.9	95.7	95.6	92.4
Darkness										
Volatiles (CO_2)	na	na	na	na	na	nd	nd	nd	nd	0.6
Flumioxazin	92.7	46.6	19.0	9.7	na	nd	nd	nd	nd	nd
482-HA	4.8	51.6	80.7	95.6	na	93.0	91.0	86.7	86.8	72.7
THPA	nd	nd	nd	nd	na	nd	nd	1.1	3.3	10.3
482-PHO	nd	nd	nd	nd	na	nd	nd	nd	nd	nd
PHO-HA	nd	nd	nd	nd	na	nd	nd	nd	nd	nd
Other unknowns	0.4	0.4	0.8	0.3	na	3.5	5.6	5.4	3.8	8.0
TOTAL	98.3	99.0	100.8	106.1	na	97.2	97.8	94.8	95.7	93.7

nd = not detected, na = not analysed

Mean mass balances were 80.9-106.1% at each timepoint in all incubation groups.

First order kinetics were considered to be acceptable and $DT_{50/90}$ values are shown in Tables 39 to 41. Overall, degradation of all compounds was rapid, with CO_2 and bound residues accounting for ≥48% of the recovered radioactivity after 30 days in illuminated water/sediment systems.

Table 39: First Order Dissipation and degradation rates of flumioxazin in water sediment systems

Location	System	Illumination			Darkness		
		DT_{50} (day)	DT_{90} (day)	χ^2 error (%)	DT_{50} (day)	DT_{90} (day)	χ^2 error (%)
Kasai Pond/THP	Natural water only	0.3	0.9	6.6	0.4	1.2	9.5
	Overlying water in w/s system	1.5	5.1	1.2	1.1	3.8	10.3
	Total w/s system	2.6	8.5	5.6	2.3	7.6	21.3
Kasai Pond/Phenyl	Overlying water in w/s system	0.9	3.1	3.3	2.1	6.9	4.2
	Total w/s system	1.6	5.3	11.9	3.6	11.9	21.4
Calwich Abbey Lake/THP	Natural water only	0.1	0.2	2.2	0.1	0.2	12.8
	Overlying water in w/s system	0.2	0.5	0.0	0.2	0.5	3.9
	Total w/s system	0.2	0.7	5.7	0.2	0.7	19.3

Table 40: First Order DT_{50} values of flumioxazin metabolites in water sediment systems under illumination

Location	System	Flumioxazin +482-HA	THPA	APF	482-PHO + PHO-HA	r^2
Kasai Pond/THP	Natural water only	2.7	38.4	na	12.4	0.986
	Overlying water in w/s system	2.2	0.6	na	10.2	0.972
	Total w/s system	4.0	1.1	na	7.1	0.985
Kasai Pond/Phenyl	Overlying water in w/s system	2.1	na	0.1	4.9	0.994
	Total w/s system	3.4	na	0.6	3.2	0.992
Calwich	Natural water only	2.3	18.2	na	2.5	0.977

Abbey Lake/THP	Overlying water in w/s system	2.1	2.7	na	1.8	0.921
	Total w/s system	3.0	4.3	na	2.3	0.962

Table 41: First Order DT_{50} values of flumioxazin metabolites in water sediment systems in darkness

Location	System	Flumioxazin	482-HA	THPA	APF	r^2
Kasai Pond/THP	Natural water only	0.4	38.7	87.5	na	0.991
	Overlying water in w/s system	1.4	3.5	0.7	na	0.883
	Total w/s system	2.2	7.2	1.5	na	0.863
Kasai Pond/Phenyl	Overlying water in w/s system	2.5	2.4	na	0.3	0.834
	Total w/s system	3.7	6.4	na	0.8	0.874
Calwich Abbey Lake/THP	Natural water only	0.1	62.4	5.6	na	0.858
	Overlying water in w/s system	0.3	16.0	0.5	na	0.986
	Total w/s system	0.2	35.0	1.3	na	0.993

The degradation rate of flumioxazin in water in the absence and presence of sediment, or in light or dark conditions, is largely unaffected, indicating that sediment or light makes an insignificant contribution to dissipation/degradation (in comparison to hydrolysis). However the degradation profiles were greatly dependent on light, with 482-PHO and PHO-HA being only found in illuminated conditions. The presence of sediment decreased the amounts of all metabolites (482-HA, THPA, 482-PHO and PHO-HA) formed. THPA degraded much faster in the presence of sediment, whilst 482-HA degraded much faster in the presence of light. Overall, degradation of all compounds was rapid, with CO_2 and bound residues accounting for ≥48% of the recovered radioactivity after 30 days in illuminated water/sediment systems.

RMS Comments:
Study is accepted. Total recovery was low at one time point (30 Days) in illuminated Calwich Abbey Lake water sediment system (80.9% AR) and dark Kasai Pond water sediment system (86.2% AR).

5.1.3 Summary and discussion of degradation

Flumioxazin is rapidly hydrolysed at pH 5-9 (DT50 ≤ 5.2 days at 25 ºC). 482-HA is the first metabolite formed and this then further degrades to THPA and APF, due to sequential cleavage of the amide bonds. Some Δ1-TPA is subsequently formed as a result of the equilibrium with THPA. Aqueous photolysis studies have been conducted with both phenyl- and tetrahydrophtalimido-labelled flumioxazin. Photolytic degradation was rapid with DT50 values in the order of 1 day. 482-PHO (max. 74.6% AR) and THPA (max. 23.0% AR) were identified as major photolytic degradation products. In addition, unknown peaks were present at >10% (max. 35.1% and 16.8% AR). A significant amount of radioactivity (max. 41.3%) remained at the origin but was shown to consist of a number of different peaks. In biotic water/sediment systems at 20ºC flumioxazin (both phenyl- and tetrahydrophtalimido- labelled) degraded quickly with whole system DT50 <1.85 days and DT90 25-69 days. Non-extractable residues reached averages of 38-61% and mineralisation reached averages of 5-29% after 98 days. The metabolites THPA (+Δ1-TPA) reached 62.8% AR after 14 days in water phase, 18.6 % AR in sediment after 7 days. A metabolite APF reached 57.7% AR after 7 days. A metabolite (SAT 482-HA-2) was found in small amounts in water (max. 5.6 % after 98 d) and sediment (max. 7 % after 60 d). The metabolite SAT-482-HA-2 also reached 20.6% AR in an anaerobic aquatic system, in the total system. Metabolites 482-HA, 482-CA and IMOXA were all identified, but at <5% AR as the average of the TLC and HPLC results. Two transient unknown compounds, Unk 23.8 and Unk 5.5, were each found >10% in one system at one timepoint but could not be identified despite extensive effort. Previous EU evaluation (7470/VI/98 rev 9) confirmed that these did not require identification since ecotoxicological risk assessment could be undertaken in the absence of identification. Metabolites requiring risk assessment in aquatic compartments were deemed to be; 482-HA, THPA and APF.

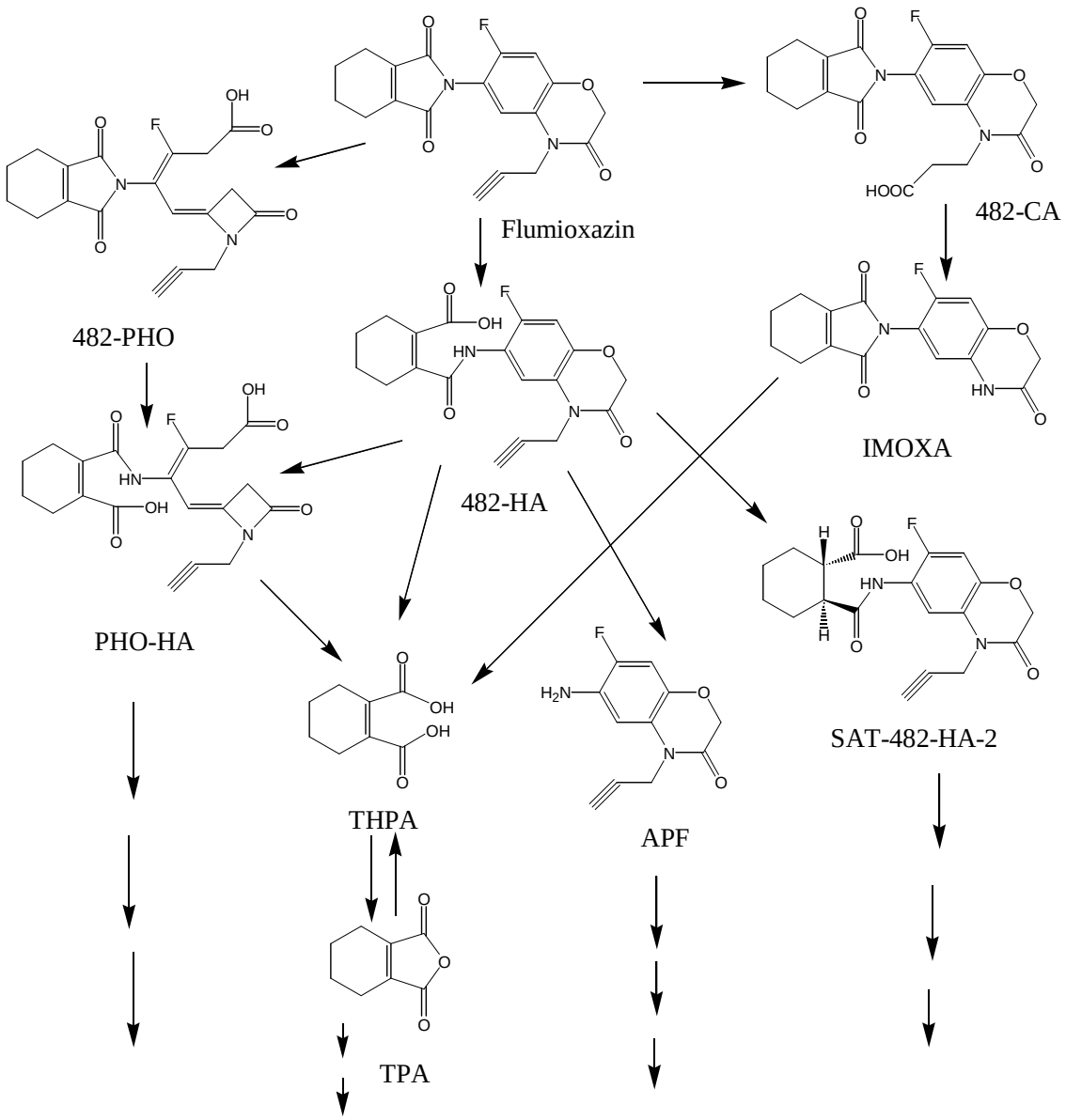
The additional data submitted in the current dossier confirm that flumioxazin is not readily biodegradable within the terms of OECD 301.

The available water sediment studies have been reassessed using FOCUS (2006) kinetics approaches (Jarvis & Mamouni, 2011b). The best fit kinetics in the whole system is DFOP, DT50 values were calculated from DT90 (DT90/3.32) with the geometric mean value of 21.6 days. As no reliable DegT50 values have been obtained for water and sediment, the geomean whole system DT50 value was used for the water phase and the default value of 1000 days was used for the sediment phase (and the reverse), instead of 72 days (sediment) and 1.5 days (water) proposed by the Applicant.

Since the previous photolysis and water/sediment studies contained unknown metabolites at >10%, and because the rapid hydrolysis and photolysis of flumioxazin results in uncertainties regarding which metabolites may be formed under realistic conditions, a further sediment/water study under illuminated conditions has been submitted (Shibata et al, 2011). In this study all major metabolites formed have been identified and hence this directly addressed the unknown peaks from the original photolysis and water sediment studies. PHO-HA was identified in the new study and this was not identified in the previous photolysis study. The new study includes the biotic degradation processes of a water sediment/system and the photolytic degradation processes of an aqueous photolysis study and hence is considered more realistic of the natural environment than either of these studies separately. In this study the maximum levels of metabolites 482-HA, THPA, APF, 482-PHO and PHO-HA were 95.6%, 57.4%, 6%, 12.2% and 50.8% respectively. The metabolite 482-HA reached max. 20.4 % in sediment.

The calculation of PECsw and PECsed for the metabolites of flumioxazin will be based on maximum percentage formed based on the entire dataset, not just values from the new illuminated study as was proposed by the Notifier. Also, despite SAT-482-HA-2 not being found in the new study, it has been included in the residue definition for risk assessment based on its presence in the original anaerobic aquatic study (Archer et al., 1996) at 20.6%. All of these metabolites have been shown to degrade rapidly in illuminated water/sediment systems, with the exception of SAT-482-HA-2. SAT-482-HA-2 appears to have been previously formed only from 482-HA and this has been shown to degrade predominantly to THPA and APF in the new illuminated water sediment study.

Figure 15: Proposed aquatic degradation routes of Flumioxazin in natural conditions



5.2 Environmental distribution

Environmental distribution of flumioxazin was determined in batch sorption study, for the soil compartment, and in water/sediment study. For the air compartment the distribution was estimated on the basis of saturated vapour pressure value and Henry´s law constant.

5.2.1 Adsorption/Desorption

Study 1 (EU monograph, 1997):
Guideline : Draft OECD screening method. Study performed in compliance with GLP. The study is valid.
Cross reference : IIA.7.1.2/01, McKay, 1994c
Due to the high susceptibility of flumioxazin to hydrolysis, the soil adsorption-desorption coefficient (log K_{OC}) was determined by high performance liquid chromatography (HPLC) method.
Flumioxazin was dissolved in a mobile phase - methanol : 0.01 M citrate buffer at pH 6.0 (55 : 45 % v/v) - and injected onto a hypersil 10 µ ODS 5S2-S565, 25 cm x 4.6 mm column and the retention time was determined four times at two concentrations (0.08 and 0.8 mg/ml). A total of seventeen reference compounds (benzamide, naphtalene, trifluraline ...) of known soil adsorption coefficients (log Koc = 1.26, 2.75, 3.94 ...) were used to calibrate the column for the soil adsorption coefficient against the capacity factor (log K). Formamide was used as a non-retained reference standard. The soil adsorption-desorption coefficient of flumioxazin was determined from the calibration graph derived from the reference standards.
The mean retention time for flumioxazin was 5.13 min. (5.10-5.15). The mean adsorption-desorption coefficient (log K_{OC}) of flumioxazin was found to be 3.15 (95 % confidence range of 2.88-3.46). The corresponding mean value of Koc is 1412 (range 758 - 2884).
On that basis, flumioxazin is expected to have low potential for mobility.

Study 2 (Addendum, 2000): Lewis C.J. (1999), report 1531/10-D2142, GLP, based on OECD guideline 106 (May 1981), acceptable.
Methods: Samples (5 g) of 3 soil types were equilibrated with 10 ml 0.01 M $CaCl_2$ for 24 h at 20° C before centrifugation. Supernatants were discarded and replaced by 20 ml 0.01 M $CaCl_2$, and a small volume (17.5 µl) of stock solution of phenyl-^{14}C flumioxazine (> 98 % radiochemical purity) prepared in acetonitrile was added to each sample to give a concentration of 0.5 mg/L. Samples were removed after 4 and 24 h and centrifuged. Supernatants were acidified (to increase the stability of flumioxazine) and analysed by LSC and HPLC on the same day. Concentrations of flumioxazine in the liquid phase were calculated. Soils were extracted with acetone-water and then with acetone-0.1N HCl. Extracts were analysed by LSC and HPLC, and concentrations of flumioazine on the soil phase were calculated taking into account water surrounding the soil after removal of the supernatant. Extracted soils were combusted. Adsorption of RA on flasks was checked.

Table 42: Soil characteristics

Soil type (origin)		Sandy loam I (UK)	Sandy loam II (UK)	Clay (UK)
Texture	sand (%)	51	74	36
	silt (2-63 µm) (%)	38	13	28
	clay (%)	11	13	36
OC (%)		2.5	1.3	1.8
pH water / 1M KCl		7.1 / 6.8	5.5 / 4.8	6.3 / 5.3
CEC mEq/100 g		16.3	8.8	15.8

Table 43: Adsorption of flumioxazine on soil

		% of applied RA (mean of 2 repl.)				Cw	Cs	Kd	Koc
		W (flumio)	Soil	Bound	Recov.	(µg/ml)	(µg/g)		
Sandy loam I	4 h	19.4 (15.7)	74.3	0.7	94.3	0.0803	1.437	17.9	716
	24 h	23.1 (10.7)	68.5	1.8	93.3	0.0544	1.337	24.6	983
Sandy loam II	4 h	26.6 (24.7)	67.0	0.6	94.2	0.1249	1.285	10.3	791
	24 h	23.5 (20.3)	65.9	2.4	91.7	0.1033	1.270	12.3	945
Clay	4 h	28.0 (25.7)	66.1	0.4	94.5	0.1312	1.230	9.4	521
	24 h	25.1 (18.3)	65.7	3.0	93.8	0.0940	1.250	13.3	739

Results: RA was not adsorbed on flasks and recoveries were acceptable. RA in water was in the range 19.4 - 28.0 % of that applied and showed little change with time. Flumioxazine was 46.3 - 92.8 % (mean 78.5 %) of the RA in water and concentrations decreased with time. Several degradation products were detected in water but were not identified. In accordance with degradation studies, they accounted for low proportions of the applied RA (1.9 - 12.4 %, mean 5.0 %). RA on soil was 66.1 - 74.3 % as flumioxazine (no metabolites were detected) and it showed little change with time. Unextractable RA was negligible (< 3 %). Flumioxazine was strongly adsorbed on soil with Kd in the range 10.3 - 17.9 after 4 h and 12.3 - 24.6 after 24 h. Koc values were calculated to be 521 - 791 (mean 676) after 4 h and they increased up to 739 - 983 (mean 889) after 24 h. Adsorption was higher in the soil with the highest OC content but it did not depend on soil pH.

Conclusions: Flumioxazine is strongly adsorbed on 3 UK soils (OC 1.3 - 2.5 %, pHw 5.5 - 7.1) with Kd in the range 12.3 - 24.6 and Koc in the range 739 - 983 (mean 889) for a 24 h equilibrium period. Because flumioxazine is not stable in water, adsorption measurement requires qualitative analysis of both water and soil phases and thus Kd values were determined for only one high initial concentration (0.5 mg/l, close to half of water solubility) for analytical reasons. However, adsorption is deemed to be sufficiently characterized because the provided data are likely to cover adsorption at lower concentrations which is expected to be more important (for neutral compounds adsorption isotherms are usually not linear - $1/n < 1$). Results are in accordance with the estimated Koc value of 1412 mL/g (CI 758-2884). They confirm the low mobility of flumioxazine in soil and the low risk for ground water contamination.

RMS Comments:
Three soil types have been tested but 4 soil types have to be tested according to a current EC regulation. These data were considered sufficient during the previous EU evaluation.

The adsorption and desorption of the active substance was conducted under OECD guideline 106 (May 1981). The main deviations from the current OECD guideline 106 (2000) are:

- Test soils do not fit guideline soils. Selection of soils from seven soil types with defined pH, %OC, % clay, and soil texture are preferred. 5 soil types are specified for screening test versus 3 tested.
- Only one concentration of flumioxazin was tested not the five recommended test substance concentrations. Freundlich adsorption isotherms were not determined.

Because of only three soil types tested (data gap for one soil type), the lowest Koc value of 739 mL/g together with the Frendlich exponent of 1.0 will be used for the risk assessment.

5.2.2 Volatilisation

Flumioxazin is not a fumigant. Its vapour pressure is 3.21 x 10^{-4} Pa at 22° C and the Henry law constant value is 1.45 x 10^{-1} Pa m^3 mol^{-1}. Normal use of flumioxazin is not expected to result in significant concentrations in air. On that basis determination of the rate and route of degradation in air is not required.

Assuming a 12-h daytime hydroxyl radical concentration of 6 x 10^5 molecules.cm^{-3}, the half-life was to be 5.84 hours.

Data/information to address this point were presented in the dossier submitted in 1994 for first inclusion in Annex I and were deemed acceptable following evaluation and peer review at EU level. No new data are presented.

RMS comments: According to FOCUS Air report (document SANCO/10553/2006 Rev 2 June 2008) flumioxazin has the potencial to reach the air Vp $\geq 10^{-4}$ Pa. Short range transport via Step 4 atmospheric deposition into surface water is not required because mitigation measures are not needed. Flumioxazin has no potencial for long range transport (DT50 < 2 days).

RMS made a request in the June 2013 reporting table: 1(32) Vol. 3, B.2.1.19, Atkinson calculation: *"RMS will prefer an update of calculation even if the new value is below the trigger 2 days for long-range transport"*. Therefore the notifier has repeated the calculations and report SBP-0062 is available, which gives a half-life of 2.26 hours using the AOPWIN v1.92 model. This report will be submitted to ECHA during the public commenting period, according to the procedure.

5.2.3 Distribution modelling

Not performed

5.3 Aquatic Bioaccumulation

Table 22: Summary of relevant information on aquatic bioaccumulation

Method	Results	Remarks	Reference
Not applicable	No experimental data are available.	Not applicable	Not applicable

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

The octanol-water partition coefficient (log Pow) of flumioxazin is 2.55 at 20°C. The flumioxazin is also rapidly hydrolysed in aqueous solution (DT_{50}: 3-5 days, 19 - 26 hours and 14 - 23 minutes at pH 5, 7 and 9, respectively). The 3 major hydrolytic degradation products : 482-HA : 7-fluoro-6-[(2-carboxyl-1-cyclohexenoyl)amino]-4-(2-propynyl)-1,4-benzoxazin-3-(2*H*)- one, APF : 6-amino-7-fluoro-4 -(2-propynyl)-1,4-benzoxazin-3-(2*H*)-one and THPA : 3, 4, 5, 6-tetrahydrophthalic acid have calculated log Pow values of 0.804, 0.127 and 0.88 respectively. These data indicate that

flumioxazin and its major hydrolytic degradation products are unlikely to partition into fatty tissues and, therefore, it is considered unnecessary to conduct a bioaccumulation study in fish.

5.3.1.2 Measured bioaccumulation data

No experimental data are available.

5.3.2 Summary and discussion of aquatic bioaccumulation

The data available indicate that flumioxazin and its major hydrolytic degradation products are considered to have a low bioaccumulation potential.

5.4 Aquatic toxicity

Table 23: Summary of relevant information on aquatic toxicity

Method	Test organism	Test system	Results			Remarks	Reference
			Endpoint	LC_{50}/EC_{50} [mg/L]	NOEC [mg/L]		
EPA 72-1	*Oncorhynchus mykiss*	Flow-through 96 h	mortality	2.3	0.92	mm	Takimoto *et al.*, 1989b
EPA 72-1	*Lepomis macrochirus*	Flow-through 96 h	mortality	> 21	3.9	mm	Takimoto *et al.*, 1989a
OECD 204	*Oncorhynchus mykiss*	Flow-through 21 d	weight reduction	> 1.2	0.37	mm	Sword *et al.*, 1992
EPA 72-2	*Daphnia magna*	Flow-through 48 h	immobilization	5.9	8.54	mm	Reed *et al.*, 1992
EPA 72-4	*Daphnia magna*	Flow-through 21 d	reproduction	-	0.057	mm	Drottar *et al.*, 1994
OECD 211	*Daphnia magna*	Semi-static 21 d	growth reduction	-	0.1	nom	Cafarella, 2000
ASTM E 1398-94	*Chironomus riparius*	Static 23 d	emergence, survival	-	0.73 (mg a.s./kg sediment)	im	Mattock D. (1997)
OECD 201	*Pseudokirchne-riella subcapitata*	Static 72 h	cell number inhibition	0.000852	0.000383	im	Blasberg *et al.*, 1992
EPA 122-2, 123-2	*Navicula pelliculosa*	Static 120 h	cell number inhibition	0.0015	< 0.000042	im	Hoberg, 1996a
EPA 122-2, 123-2	*Lemna gibba*	Semi-static 14 d	biomass reduction	0.00035	0.000051	im	Hoberg, 1996b

mm – mean measured concentration
im – initial measured concentration
nom – nominal concentration

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

Acute toxicity to bluegill sunfish

Reference: Takimoto Y., Hagino S., Saito S. (1989a), Study number SBW-90-0002 - OLD STUDY (DAR 1997, Addendum 2000)
Test guideline: FIFRA, Subdivision E 72-1
GLP compliance: yes
Test item: S-53482 (purity 94.8%)
Lot No./Batch No.: PYG-89021-M

The acute toxicity of flumioxazin to bluegill sunfish (*Lepomis macrochirus*) has been determined over 96 hours in a flow-through system at a temperature of 22 °C.

Groups of ten fish were exposed to various concentrations of flumioxazin, as a solution in N,N-dimethylformamide and polyoxyethylene hydrogenated castor oil, diluted in distilled water. A control group of fish was exposed to the solvent vehicles alone. Dissolved oxygen, pH, temperature and test solution concentrations were measured at regular intervals throughout the study. The fish were observed for mortalities and symptoms of toxicity after 24, 48, 72 and 96 hours of exposure.

Due to low solubility of the a.s., testing was performed using a suspension in a mixture of 50% DMF and 50% polyoxyethylene hydrogenated castor oil (HCO-40). The solvent concentration in water for all test and control groups was 3.2 g/l, *i.e.*, 32-fold higher than the acceptable level (100 mg/l). Mean measured concentrations were in the range 52-70% of nominal (nominal test values: 3.2, 5.6, 10, 18 and 32 mg/l), therefore the results are based on mean measured concentrations. The dissolved oxygen concentration in the water was greater than 7.26 mg/l at all times and the pH values were in the range 7.06 - 7.44.

There were no mortalities in any of the groups throughout the study. Symptoms of toxicity were seen in fish exposed to concentrations of 6.3 mg/l and above. These symptoms included abnormal respiration and swimming at the surface. It was concluded that the 24, 48, 72 and 96 hour LC_{50}'s of flumioxazin to bluegill sunfish were all greater than 21 mg/l. The "no-effect" concentration determined in this study was 3.9 mg/l.

RMS Comment (DAR 1997)

Study conducted in compliance with GLP. The study is valid. The flumioxazin is of slight acute toxicity to bluegill sunfish.

RMS Comment (Addendum 2000)

It is agreed by the Rapporteur that very high concentrations of solvent were used in an unsuccesful attempt to overcome the intrinsic low solubility of the substance (1.79 mg/l). However, a) no mortality was observed in both solvent and water controls, whatever the dose, and b) there is no reason to assume that a lower level of solvent could lead to a higher toxicity of the compound.

RMS Comment (2012)

The reported study is GLP compliant and was conducted according to FIFRA 72-1 guideline. The test results are in compliance with the guideline's validity criteria. The study is acceptable for regulatory use.

Acute toxicity to rainbow trout

Reference: Takimoto Y., Hagino S., Saito S. (1989b), Study number SBW-90-0001- OLD STUDY (DAR 1997)
Test guideline: FIFRA, Subdivision E 72-1
GLP compliance: yes
Test item: S-53482 (purity 94.8%)
Lot No./Batch No.: PYG-89021-M

The acute toxicity of flumioxazin to rainbow trout (*Oncorhynchus mykiss*) has been determined over 96 hours in a flow-through system at a temperature of 13 °C.

Groups of ten fish were exposed to various concentrations of flumioxazin, as a solution in N,N-dimethylformamide and polyoxyethylene hydrogenated castor oil, diluted in distilled water. A control group of fish was exposed to the solvent vehicles alone. Dissolved oxygen, pH, temperature and test solution concentrations were measured at regular intervals throughout the study. The fish were observed for mortalities and symptoms of toxicity after 24, 48, 72 and 96 hour LC_{50} values were calculated by probit analysis.

The mean measured concentrations for the test solutions were in the range of 91 - 111 % of the nominal concentrations. The dissolved oxygen concentration in the water was greater than 8.3 mg/l at all times and the pH values were in the range 7.0 - 7.3. The water temperatures were in the range 12.6 - 12.9 °C. Mortalities and symptoms of toxicity were seen in fish exposed to concentrations of 2.0 mg/l and above. The symptoms of toxicity included swimming at the surface, swimming at the bottom of the tank, loss of equilibrium and lethargy. The highest concentration causing no mortalities was 0.92 mg/l and the lowest concentration causing 100 % mortalities was 5.4 mg/l. There were no mortalities or symptoms of toxicity in the solvent control group. The 24, 48 and 72 hour LC_{50}'s of flumioxazin to rainbow trout are 2.9 – 5.4 mg/l and 96 hour LC_{50} is 2.3 mg/l, based on mean measured concentration. The 96 hour "no-effect" concentration for flumioxazin determined in this study was 0.92 mg/l, based on mean measured concentration.

RMS Comment (DAR 1997)

Study conducted in compliance with GLP. The study is valid. The flumioxazin is of moderate acute toxicity to rainbow trout.

RMS Comment (2012)

The reported study is GLP compliant and and was conducted according to FIFRA 72-1 guideline. The test results are in compliance with the guideline's validity criteria. The study is acceptable for regulatory use.

5.4.1.2 Long-term toxicity to fish

Chronic toxicity test on juvenile fish rainbow trout

Reference: Sword M. C. *et al.* (1992), Study number SBW-21-0009 - OLD STUDY (DAR 1997)
Test guideline: OECD 204
GLP compliance: yes
Test item: S-53482 (purity 94.3%)
Lot No./Batch No.: PYG-89021-M

The chronic toxicity of flumioxazin to rainbow trout (*Oncorhynchus mykiss*) has been determined over 21 days in a flow-through system at a temperature of approximately 15-16°C with a 16 : 8-hour light-dark cycle.

Groups of twenty fish were exposed to various concentrations of flumioxazin, as aqueous dilutions of a solution in dimethylformamide (DMF). The concentrations of flumioxazin in the test solutions, temperature, dissolved oxygen and pH were measured at regular intervals throughout the study. The fish were observed for mortalities and symptoms of toxicity at various time intervals and the LC_{50} values were calculated by the method of Stephan. The lengths and weights of fish surviving to the end of the study were recorded and compared with the control values.

The mean concentrations of the stock solutions was 98 % of the nominal concentration and the mean measured concentrations of the diluted solutions were in the range of 60 - 80 % of the nominal concentrations. Therefore the results are based on mean measured concentrations. Dissolved oxygen concentration were in the range of 8.8 - 9.5 mg/l (representing 91 - 98% saturation, respectively) and the pH of the solutions ranged from 7.9 - 8.3. There were no mortalities in fish exposed to flumioxazin at concentrations of up to 0.37 mg/l. There were 10, 10 and 60 % cumulative mortality in the groups exposed to flumioxazin at concentrations for 0.61, 1.2 and 2.4 mg/l, respectively. Fish exposed to concentrations of 0.61 mg/l and above exhibited reduced food consumption. The most common signs of toxicity were surfacing, loss of equilibrium, dark discoloration, fish on the bottom of the test chamber, laboured respiration, vertical orientation, erratic swimming and/or quiescence. There was no mortality or abnormal effects in the negative control, solvent control or groups exposed to flumioxazin at concentrations of 0.20 and 0.347 mg/l. Statistical analysis indicated no significant reduction in survival in groups exposed to flumioxazin at concentrations of up to 1.2 mg/l. There was a statistically significant reduction in the mean weights of fish surviving to 21 days which were exposed to concentrations of 0.61 and 1.2 mg/l when compared to the pooled control groups. The threshold level for observed effects was 0.61 mg/l.

It was concluded that the 21 day LC_{50} of flumioxazin to rainbow trout was greater than 1.2 mg/l and the 21 day "no-observed-effect" concentration in this study was found to be 0.37 mg/l.

RMS Comment (DAR 1997)

Study conducted in compliance with GLP. The study is valid.

RMS Comment (2012)

The reported study is GLP compliant and and was conducted according to OECD 204 guideline. The test results are in compliance with the guideline’s validity criteria. The study is acceptable for regulatory use.

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

Acute toxicity to *Daphnia magna*

Reference: Reed D., Swigert J.P. (1992), Study number SBW-21-0007 - OLD STUDY (DAR 1997, Addendum 2000)
Test guideline: FIFRA, Subdivision E Series 72-2
GLP compliance: yes
Test item: S-53482 (purity 94.7%)
Lot No./Batch No.: PPG-90111-M

Methods: Duplicate test chambers each containing ten *D. magna* were exposed to a series of concentrations of flumioxazin in a 1:1 mixture of N,N-dimethylformamide (DMF) and polyoxyethylene hydrogenated castor oil (HCO-40). A flow-through system was used at a temperature of 20 °C with a 16 : 8 hour, light : dark cycle. The concentration of flumioxazin in the test solutions was measured at regular intervals throughout the study. Duplicate analyses were performed on samples that had been centrifuged and non-centrifuged. Dissolved oxygen and pH was measured at 24 hour intervals and the temperature was measured at the beginning and end of the study.

The effects of the substance on the *Daphnia* were assessed at 18, 24 and 48 hours and the LC_{50} values were calculated by the method of Stephan. Negative and solvent control groups were tested simultaneously. There were considerable differences between the nominal, centrifuged and non centrifuged measured concentrations in the test solutions. These differences were related to the low water solubility, the presence of precipitated material and the rapid degradation of flumioxazin under slightly basic conditions. The LC_{50} values were, therefore, calculated using the measured concentrations of both the centrifuged and non-centrifuged samples. The dissolved oxygen concentrations ranged from 7.8 - 8.2 mg/l and the pH remained constant between 8.1 and 8.4.

It is reported in the study (page 9) that, due to low solubility of the a.s., testing was performed using a suspension in a mixture of 50% DMF and 50% polyoxyethylene hydrogenated castor oil (HCO-40). The concentration of the DMF/HCO-40 mixture was 4 ml/l in all treatment levels, in water control and in solvent control groups. The nominal concentrations were 15.6, 25.9, 43.2, 72, and 120 mg/l.

Visual observation showed that a large amount of precipitate occurred in groups treated at higher doses. It was assumed that the immobility observed in some groups was likely the result of the "mechanical" interaction with the precipitated material. Water samples were collected: a part was centrifuged prior to analysis to remove the precipitated material (5 min x 2 000 rpm) and the other part was analysed without prior centrifugation. The results show differences between the nominal and measured values, these differences increasing in parallel with the increase in nominal concentrations. Finally, very high variability occurred in samples of water from groups treated at the highest doses. However, if the lowest dose is considered (*i.e.*, 15.6 mg/l), percent of nominal values were in the range 20-31% of nominal in the 6 centrifuged samples and 27-33% of nominal in the non-centrifuged samples. The mean values were 3.74 and 4.58 mg a.s./l for centrifuged and non-centrifuged samples, respectively. Only 10% immobilisation was observed at this dose.

Mortality ranged from 15 % in the group exposed to the highest concentration, to 0 - 10 % in groups exposed to lower concentrations of flumioxazin. Immobilisation ranged from 0 - 85 % in the treated groups. Combined immobilisation and mortality in the negative and solvent controls was 5 and 10 %, respectively. A dose-related increase in the combined mortality and immobilisation data was noted

when compared to the concentrations of flumioxazin in the non-centrifuged samples. However, no dose-response relationship existed with the concentrations of flumioxazin in the centrifuged samples. Immobilisation was considered to be a result of a "mechanical" effect of the precipitated material rather than due to substance toxicity. The lowest concentration causing 100 % immobilisation and mortality was 73.46 mg/l (concentration in non centrifuged samples). The 24 and 48 hour EC_{50}'s are presented in Table B.9.2.1. The effects on mobility and mortality seen in the groups exposed to concentrations of 3.74 and 8.54 mg/l (non centrifuged concentrations) were comparable to those seen in the solvent control and, therefore, 8.54 mg/l was considered to be a "no-observed-effect" level for flumioxazin to *Daphnia*.

Table B.9.2.1 EC50 values

Time	EC50, mg a.s./L (95% confidence limits)	
	Centrifuged sample	Non-centrifuged sample
24 h	>9.25[1]	>73.46[1]
48 h	5.9 (5.4 – 6.5)[2]	17 (14 – 22)[2]

[1] insufficient effects at 24 h precluded the calculation of an EC50
[2] EC50 calculated by probit method

RMS Comment (DAR 1997)

Study conducted in compliance with GLP. The study is valid.

RMS Comment (Addendum 2000)

It appears that the a.s. is very difficult to disperse in water even after solubilisation in common solvents such as methanol, acetone triethylene glycol and dimethylformamide (see Takimoto (1989a), Acute effects of S-53482 on the bluegill, Study number FTX-89006).
It is believed by the Rapporteur that the conditions of centrifugation are not sufficiently effective to remove all light suspended/dispersed material. Centrifugation at higher speed could lead to values close to the solubility of the a.s in water (including solvents) and it was estimated (page 15 of the study) that the solubility of the substance in the presence of a concentration of emulsifiers of 4 mg/l was probably in the range of 4-6 mg/l. In these conditions, it is considered that the calculation of a relevant EC50 is not possible, as it is recognised in Guideline OECD 202. The EC50 value of 5.9 mg/l which was tentatively proposed in the study has to be considered as a rough estimate. This value was not considered as "irrelevant" by the Rapporteur and was used in a provisional risk assessment.

RMS Comment (2012)

The reported study is GLP compliant and and was conducted according to FIFRA 72-4 guideline, which is in line with the current OECD 202 guideline. The test results are in compliance with the guideline's validity criteria. Given the solubility problems, the study is acceptable for regulatory use with limitation.

5.4.2.2 Long-term toxicity to aquatic invertebrates

Study 1: Chronic toxicity to *Daphnia magna*

Reference: Drottar K.R., Swigert J.P. (1994), Study number SBW-41-0014 - OLD STUDY (DAR 1997, Addendum 2000)
Test guideline: FIFRA, Subdivision E Series 72-4
GLP compliance: yes

Test item: S-53482 (purity 94.8%)
Lot No./Batch No.: PYG-89021-M

Methods: A study was performed to evaluate the effects of the flumioxazin on the survival, growth and reproduction of *Daphnia magna* during a 21 day exposure period under flow-through test conditions. Nominal concentrations of flumioxazin (and [Ph-^{14}C]-flumioxazin) applied were 25, 50, 100, 200 and 400 µg a.s./l. Mean measured concentrations of flumioxazin were 15, 28, 57, 107 and 205 µg/l. Mean measured [Ph-^{14}C]-flumioxazin concentrations determined by LSC ranged from 93 to 101 % of nominal values, indicating that the delivery system was working properly. As a result, mean measured concentrations were 24, 49, 101, 196 and 372 µg flumioxazin equivalent/l. Temperature was at 20 ± 1 °C, dissolved oxygen concentration exceeded 60 % of saturation and pH ranged at 8.2 - 8.5.

It is reported in the study (page 10) that "the concentration in the treatment and solvent group was approximately 0.1 ml/l". Working stock concentrations were prepared with 0.0264, 0.527, 1.06, 2.11, and 4.22 mg a.s./ml solvent (DMF). To obtain the nominal concentrations which were used in this study (25, 50, 100, 200 and 400 microg a.s./l of water), a dose of 0.095 ml of these working solutions per litre of water was used. The concentration of adjuvant is then slightly lower than the threshold level indicated in OECD 202Guideline (100 mg/l, *i.e.*, *ca* 0.1 ml of solvent/l).

Results:

Effects at 21 d are indicated in Table B.9.2.2.

Table B.9.2.2 Effects of flumioxazin on *Daphnia magna* after 21 days

Nominal concentrations (microg/l)	Negative control	Solvent control	25	50	100	200	400
Measured concentrations (microg/l)	0	0	15	28	57	107	205
Immobilisation (%)	9	14	14	0	5	55	73
Neonate production *(in brackets: CV's)*	60 (28.5%)	51 (34%)	45 (37.1%)	39 (55.4%)	37 (37.2%)	0	0

After 21 days of exposure, survival was 91 and 86 % in the negative control and solvent control, respectively. In the treatment groups, survival ranged from 100 % at 28 µg a.s./l to 27 % at 205 µg a.s./l. Survival was significantly reduced in both 107 and 205 µg a.s./l treatments groups in comparison with solvent control. Consequently, the no observed effect concentration (NOEC) for survival was 57 µg a.s./l.

Adult daphnids produced an average of 60 and 51 neonates per adult in the negative and solvent controls, respectively, with no statistical differences. The reproduction was not significantly reduced in the 15, 28 or 57 µg/l treatment groups. The within group variability (RSD = 28 and 34 % in both control groups) do not seem to be unreasonably high. The mean carapaces length and mean dry weight of adult daphnids in the negative and solvent control groups were 4.0 mm / 0.51 mg and 4.1 mm / 0,45 mg respectively, with no statistical differences. Length and weight were not significantly reduced in the 15, 28 or 57 µg/l treatment groups.

In conclusion, there is no apparent treatment-related effects upon survival growth or reproduction of *D. magna* exposed to a 57 µg a.s./l. Reproduction was the most sensitive biological parameter measured in the chronic toxicity test. The no observed concentration was 57 µg a.s./l and the lowest observable effect concentration was 107 µg a.s./l, based on mean measured concentrations.

RMS Comment (DAR 1997)

Study conducted in compliance with GLP. The study is valid.

RMS Comment (Addendum 2000)

Analyses of reproduction, length and weight data were conducted using statistically relevant methods. Some variability occurred in the number of neonates per female in negative and solvent control groups (CV's equal to 28 and 34%, respectively). No indications are provided in the guideline OECD 202 about acceptable values. However, it is considered that a high variability *intra*-groups could lead to falsely accept the null hypothesis (*i.e.*, no difference between groups). Study was conducted according to FIFRA Guidelines, not to OECD 202, thus the number of replicates in each group is different (7 in the study *vs* 10 in OECD). Furthermore, reproduction success is low in negative and solvent control groups (60 and 51 neonates for 21 d, respectively).

RMS Comment (2012)

The reported study is GLP compliant and and was conducted according to FIFRA 72-4 guideline, which is in line with the current OECD 211 guideline. The test results are in compliance with the guideline's validity criteria (the mortality of the parent animals should not exceed 20% at the end of the test in control; the mean number of offspring per female in control should be ≥60). The study is acceptable for regulatory use.

Study 2: Chronic toxicity to *Daphnia magna*

Reference: Cafarella M.A. (2000), Study number 13048.6202 (SBW-0050) – OLD STUDY (Addendum 2000)
Test guideline: OECD 211
GLP compliance: yes
Test item: S-53482 (purity 99.0%)
Lot No./Batch No.: 50721

Methods: Flumioxazine (99%) was dosed at 0.05-0.8 mg/l to 10 individual daphnids per concentration, along with acetone (0.01%) and water controls. Stock solutions and test solutions were renewed every 3 d. Adult survival, reproduction and growth were observed for 21 d.

Samples were analysed by HPLC/UV for the detection of the primary hydrolysis product 482-HA due to the rapid hydrolysis of flumioxazine at basic pH. Further degradation of 482-HA to APF and THPA was determined at the highest test concentration by HPLC/UV. Flumioxazine was analysed by GC/ECD method immediately after fortification and in aged solutions.

Results: The water quality parameters were unaffected by the concentrations of flumioxazine and remained within acceptable ranges for the survival and reproduction of daphnids. The pH measures ranged within 7.6 to 8.5.

The recoveries of flumioxazine from the exposure solutions immediately after the fortification were approximately 90% and ranged between 79 and 91% of the nominal values. After 3 d, the recoveries

of flumioxazine from exposure solutions were all less than the limit of quantification. Flumioxazine and 482-HA analyses showed that most of the parent had hydrolysed to 482-HA during the 30 min of stirring the exposure solutions prior to conducting the renewal. Further degradation of 482-HA was minimal (i.e., < 10%) between the 3 d renewal intervals, indicating that 482-HA was generally stable under test conditions. Thus, the quantitation of flumioxazine was based on the detection of 482-HA and equated to a measured concentration of flumioxazine. The measures obtained for the new freshly-prepared solutions generally ranged between 80 and 100% of nominal values, while the aged solutions generally ranged between 70 and 80% of nominal values. In conclusion, due to rapid hydrolysis of flumioxazine to 482-HA at a basic pH, it is likely that the daphnids were exposed primarily to the hydrolysis product throughout the 21 d test period. Therefore, statistical analysis for all biological endpoints and treatment effects are based on nominal concentrations of flumioxazine.

The 21 d EC50 was estimated by non-linear interpolation to be 0.37 mg as/l with 95% confidence limits of 0.2 and 0.8 mg as/l (calculated by binomial probability). Although significant, the reduced total body length observed at 0.05 mg/l was considered to be a statistical anomaly, since it was not part of a dose-response and the small 2.9% reduction was not considered biologically significant. Growth parameters are the most sensitive indicators of toxicity of flumioxazine to *D. magna* under chronic exposure.

NOEC = 0.1 mg as/l, MATC = 0.14 mg as/l, LOEC = 0.2 mg as/l (nominal concentrations)

RMS Comment (Addendum 2000)
Acceptable.

RMS Comment (2012)

The reported study is GLP compliant and and was conducted according to OECD 211 guideline. The test results are in compliance with the guideline's validity criteria (the mortality of the parent animals should not exceed 20% at the end of the test in control; the mean number of offspring per female in control should be ≥60). The study is acceptable for regulatory use.

5.4.3 Algae and aquatic plants

Toxicity to *Pseudokirchneriella subcapitata* (formerly named *Selenastrum capricornutum)*

Reference: Blasberg J. *et al*. (1992), Study number SBW-21-0008 - OLD STUDY (DAR 1997)
Test guideline: OECD 201
GLP compliance: yes
Test item: S-53482 (purity 94.3%)
Lot No./Batch No.: PYG-89021-M

Triplicate flasks of *Selenastrum capricornutum* were exposed to flumioxazin in acetone at nominal concentrations of 0.54, 1.1, 2.1, 4.3 and 8.5 µg/l. The concentration of flumioxazin in the test solutions were measured at the beginning and end of the study by gas-liquid chromatography (GLC). The algae were cultured under continuous light at 24 °C for up to 72 hours. Acetone was tested as a solvent control. Temperature and pH were measured at the beginning and end of the study. Samples were taken at 0, 24, 48 and 72 hours for measurement of cell densities. The effect of flumioxazin on the inhibition of growth of the algae was assessed from the cell densities by statistical analysis and the EC_{10}, EC_{50} and EC_{90} values for inhibition were calculated from these data.

Initial mean measured concentrations of flumioxazin in the test solutions were 71 % of the nominal concentrations. However, by the end of the study, the concentrations in the test solutions were much lower than the initial nominal concentrations, indicating that flumioxazin was unstable under the conditions of the study. The temperature remained constant at 24 °C throughout the study and the pH ranged from 7.3 to 7.7. There were statistically significant inhibitions in growth of algae exposed to flumioxazin at nominal concentrations of 1.1, 2.1, 4.3 and 8.5 µg/l (Table B.9.2.4). The 24, 48 and 72 hour EC_{50} values for algal growth were calculated to be 1.1, 1.2 and 1.2 µg/l, respectively.

It was concluded that the 72 hour EC_{10}, EC_{50} and EC_{90} values of flumioxazin to *Selenastrum capricornutum* were 0.88, 1.2 and 1.7 µg/l, respectively, based on nominal concentrations and the "no-observed-effect" concentration in this study, based on nominal concentrations, was considered to be 0.54 µg/l.

Table B.9.2.4 Measured cell counts for *Selenastrum capricornutum* during the exposure to S-53482

	Mean Cell Counts (3 Flasks) × 10^4 cells/mL			
Nominal Test Conc. (µg/L)	0-Hour[a]	24-Hour[b]	48-Hour[b]	72-Hour[b]
Control	0.82	4.6	25	149
Vehicle Blank	0.78	4.5	25	150
0.54		4.4	23	141
1.1		*2.9	*14	*99
2.1		*0.93	*1.5	*1.7
4.3		*0.78	*1.0	*0.56
8.5		*0.56	*0.29	*0.48

[a] Values were obtained from the cell count data forms
[b] Values obtained from SAS

* Denotes a significant ($p \leq 0.05$) inhibition effect from the control and vehicle blank as calculated using transformed (square root) cell counts by Dunnett's test.

RMS Comment (DAR 1997)

Study conducted in compliance with GLP. The study is valid.

Since initial measured concentrations were 71% of nominal values, the 72 hour EC_{50} for algal growth 1.2 µg/l was recalculated by RMS (see the Reply to Danish Comments, 15 June 2000). Thus, EC_{50} is 0.852 µg/l.

RMS Comment (2012)

The reported study is GLP compliant and and was conducted according to OECD 201 guideline. The test results are in compliance with the current guideline's validity criterium (the increase of cell numbers, measured in the control between 0 h and 72 h should be greater than the criterion value of >16). The study is acceptable for regulatory use.

Since initial measured concentrations were 71% of nominal values, the 72 hour NOEC 0.54 µg/l was recalculated to be 0.383 µg/l.

Toxicity to *Navicula pelliculosa*

Reference: Hoberg J. R. (1996a), Study number 96-10-6717 (SBW-0028) - OLD STUDY (DAR 1997)
Test guideline: EPA-FIFRA 122-2, 123-2
GLP compliance: yes
Test item: V-53482 (~ S-53482; purity 99.5%)
Lot No./Batch No.: 40303

Triplicate flasks of *Navicula pelliculosa* at 1.0 x 10^4 cells/ml were exposed to flumioxazin in acetone at nominal concentrations of 0.048, 0.093, 0.19, 0.38, 0.75, 1.5 and 3.0 µg/l. The algae were cultured at 24 ± 1 ° C, under continuous light at 24 °C for up to 5 days. Acetone was tested as a solvent control. Temperature and pH were measured at the beginning and end of the study. Samples were taken at Day 0, 1, 2, 3, 4 and 5 for measurement of cell densities. The effect of flumioxazin on the inhibition of growth of the algae was assessed from the cell densities by statistical analysis and the EC_{05}, EC_{25} and EC_{50} values for inhibition were calculated from these data.

Initial mean measured concentrations of flumioxazin in the test solutions were 0.042, 0.074, 0 .15, 0.31, 0.61, 1.2 and 2.4 µg flumioxazin per litre. These concentrations were used to define the treatments. The temperature remained between 24 and 25 °C throughout the study and the pH ranged from 7.1 (test initiation) to 8.4 - 8.6 (test termination). Light intensity was 390 to 480 foot-candles and the shaking rate was 100 rpm.

Statistical analysis demonstrated a significant reduction in cell density in all treatment levels tested as compared to the control data (Table B.9.2.5). It was concluded that the 5 day EC_{05}, EC_{25} and EC_{50} values of flumioxazin to *Navicula pelliculosa* were 0.041, 0.59 and 1.5 µg/l, respectively, based on initial measured concentrations and the "no-observed-effect" concentration in this study was considered to be < 0.042 µg/l.

Table B.9.2.5 Cell density (x 10^4 cells/mL) of *Navicula pelliculosa* after 1,2,3,4 and 5 days of exposure

Initial Measured Concentration (µg A.I./L)		Observation Interval Day 1	Day 2	Day 3	Day 4	Day 5	Day 5 Percent Inhibition
Control	A	3	15	47	85	117	
	B	3	10	43	88	125	
	C	2	13	44	83	145	
Mean(SD)[a]		2(1)	13(2)	45(2)	85(2)	129(15)	NA[b]
0.042	A	1	10	43	76	118	
	B	2	9	41	84	121	
	C	4	10	42	82	117	
Mean(SD)[a]		2(1)	10(1)	42(1)	81(4)	119(2)[c]	8.1
0.074	A	3	10	41	78	119	
	B	3	10	34	81	118	
	C	3	9	40	82	115	
Mean(SD)[a]		3(<1)	9(<1)	38(4)	80(2)	117(2)[c]	9.2
0.15	A	2	9	44	75	98	
	B	3	9	43	100	109	
	C	2	7	38	79	113	
Mean(SD)[a]		2(1)	8(1)	42(3)	85(13)	107(8)[c]	17
0.31	A	2	7	36	75	111	
	B	4	8	38	79	107	
	C	3	10	35	78	110	
Mean(SD)[a]		3(1)	8(1)	36(2)	77(2)	110(2)[c]	15
0.61	A	1	4	27	65	100	
	B	2	7	26	66	96	
	C	2	5	31	60	102	
Mean(SD)[a]		1(1)	5(2)	28(2)	63(3)	99(3)[c]	23

[a] Mean, standard deviation (SD) and percent inhibition were calculated from original raw data, not from the rounded values presented in this table.
[b] NA = Not applicable
[c] Significantly reduced ($p \leq 0.05$) as compared to the control based on Williams' Test.

RMS Comment (DAR 1997)

Study conducted in compliance with GLP. The study is valid.

RMS Comment (2012)

The reported study is GLP compliant and and was conducted according to OECD 201 guideline. The test results are in compliance with the current guideline's validity criteria (the increase of cell numbers, measured in the control between 0 h and 72 h should be greater than the criterion value of >16). The study is acceptable for regulatory use.

Toxicity to *Lemna gibba*

Reference: Hoberg J. R. (1996b), Study number 96-10-6722 (SBW-0027) – OLD STUDY (DAR 1997)
Test guideline: EPA-FIFRA 122-2, 123-2.

GLP compliance: yes
Test item: V-53482 (~ S-53482; purity 99.5%)
Lot No./Batch No.: 40303

A study was performed on *Lemna gibba* G3. Five, three fronds each, plants were introduced in three vessels per treatment plus one for analysis purposes. Nominal concentrations of 0.064, 0.13, 0.25, 0.50, 1.0 and 2.0 µg/l plus one acetone control were used.

Temperature was maintained at 25 ± 1°C with a continuous light intensity of 300 to 500 foot-candles during the 14 days of the test. Observations and / or analysis were performed at days 0, 3, 6, 9, 12 and 14.

Initial mean measured concentrations of test material in the test solutions ranged from 85 to 90 % of the nominal concentrations and, at Day 3, at 23 %. The initial measured concentrations derived from LSC analysis were 0.051, 0.11, 0.22, 0.44, 0.87 and 1.7 µg/l and defined the treatment levels.

The temperature remained between 24 and 25 °C throughout the study and the pH ranged from 5.0 - 5.1 (test initiation) to 4.8 - 6.2 (test termination). Light intensity was 350 to 440 foot-candles.

Statistical analysis demonstrated a significant reduction in frond density in the 3 higher treatment levels tested as compared to the control data (Table B.9.2.14). Based on initial measured test concentrations, the 14 days EC_{50} = 0.35 µg/l (95 % confidence limits = 0.14 and 0.90 µg/l) and the 14 days no observed effect concentration (NOEC) was 0.051 µg/l flumioxazin.

Table B.9.2.14 Frond production (density) and observations recorded for *Lemna gibba* after 3, 6, 9, 12 and 14 days of exposure

Initial Measured Concentration (µg A.I./L)		Fronds/replicate					Percent Reduction (Day 14)
		Day 3	Day 6	Day 9	Day 12	Day 14	
Control	A	39	100	200	480	760	
	B	38	116	210	450	630	
	C	40	113	220	480	750	
	Mean(SD)[a]	39(1.0)	110(8.5)	210(8.1)	470(15)	710(75)	NA[b]
0.051	A	39	100	200	400	750	
	B	38	105	220	400	700	
	C	36	99	200	400	790	
	Mean(SD)[a]	38(1.5)	100(3.2)	210(12)	400(4.7)	740(48)	-4.3
0.11	A	41	98	200	390	730	
	B	34	98	200	350	750	
	C	37	95	200	360	640	
	Mean(SD)[a]	37(3.5)	97(1.7)	200(2.1)	370(21)	700(56)[c]	1.2
0.22	A	35	98	180	330	650	
	B	32	88	180	320	640	
	C	37	102	200	340	680	
	Mean(SD)[a]	35(2.5)	96(7.2)	190(8.1)	330(11)[c]	660(23)[cd]	8.1
0.44	A	35	98	180	240	330	
	B	33	84	180	240	360	
	C	31	82	180	240	320	
	Mean(SD)[a]	33(2.0)	88(8.7)[cde]	180(1.7)[cde]	240(2.0)[cd]	330(22)[cdh]	53
0.87	A	34	81	160	200	220	
	B	31	80	150	200	230	
	C	33	83	150	210	210	
	Mean(SD)[a]	33(1.5)[cd]	81(1.5)[cde]	150(6.1)[cde]	200(2.7)[cde]	220(7.4)[cdeh]	69
1.7	A	35	53	62	78	100	
	B	32	65	64	84	120	
	C	27	50	51	81	120	
	Mean(SD)[a]	31(4.0)[cd]	56(7.9)[cde]	59(7.0)[cde]	81(3.0)[cde]	110(9.7)[cfgh]	84

[a] Mean, standard deviation (SD) and percent reduction were calculated from original raw data, not from the rounded values presented in this table.
[b] NA = Not Applicable
[c] Fronds were observed to be smaller in comparison to control.
[d] Fronds were observed to have reduced root formation in comparison to control.
[e] Fronds were observed to be slightly chlorotic in comparison to control.
[f] Fronds were observed to have very little root formation in comparison to control.
[g] Fronds were observed to be chlorotic in comparison to control.
[h] Statistically reduced in comparison to control, based on Williams' Test.

RMS Comment (DAR 1997)

Study conducted in compliance with GLP. The study is valid.

RMS Comment (2012)

The reported study is GLP compliant and was conducted according to FIFRA 122-2 and, 123-22 guideline. However, the study is not in line with the current guideline OECD 221, that recommends observations on two response variables: average specific growth rate and yield. Both response

variables should be based on frond numbers and on one additional parameter of observation (frond area or dry/fresh weight). The results of the study did only allow calculation of inhibition of the two response variables (growth rate and yield) based on frond numbers - since biomass was only observed on day 14 (and no estimate of starting biomass was available). Since a higher tier study on phytoplankton and aquatic macrophytes was available, the reported study on *Lemna gibba* is considered as supplemental information.

5.4.4 Other aquatic organisms (including sediment)

Chronic toxicity to the sediment dwelling invertebrate *Chironomus riparius*

Reference: Mattock D. (1997), Study number 1531/2-1018 (SBW-0042) - OLD STUDY (DAR 1997)
Guideline: ASTM E 1398-94, DoE 3460 P2
GLP compliance: yes
Test item: S-53482 (purity 99.0%)
Lot No./Batch No.: 50721

Twenty *Chironomus riparius* larvae were used in each of eight vessels per treatment. The sediment, coming from a site known to be free of contamination, contained 3.35 % organic carbon set by dilution with sablesand; particle size distribution: clay 11.6%, silt 11.3%, sand 77.2%; texture classification: sandy loam; organic matter 4.0%. Sediment was spiked with acetone stock solutions to prepare nominal concentrations of 1.5, 0.5, 0.17, 0.06 and 0.02 mg/kg. Two control treatments: dilution water + sediment and dilution water + acetone + sediment. Aeration was given all along the test. Overlying lost water through evaporation was replaced by reverse osmosis water. Larvae were fed with ground TetraMin ™ 3 times per week during the 23 days of the test duration. Test organisms were observed daily from the onset of emergence until Day23, flumioxazin analysis was performed in overlying water at Days 0, 14 23, and in sediment at Days 0, 1, 3, 7, 14 and 23. The majority of determinations in water were at or below the limit of quantitation (0. 5 µg/l) with two exceptions: 3.9 and 22.4 µg/l were obtained for the control and 1.5 mg/kg treatment respectively. Measured concentrations in the sediment are given in the table B.9.2.3.

Table B.9.2.3 Measured concentrations in the sediment

Nominal concentration	Measured concentration (mg/kg)					
(mg/kg)	Day 0	Day 1	Day 3	Day 7	Day 14	Day 23
Control	< LQ	< LQ	< LQ	< LQ	< LQ	< LQ
Solvent control	< LQ	< LQ	< LQ	< LQ	< LQ	< LQ
0.02	0.01	0.02	0.02	< LQ	< LQ	< LQ
0.06	0.05	0.03	0.02	0.01	< LQ	< LQ
0.17	0.09	0.07	0.05	0.03	0.02	< LQ
0.5	0.22	0.19	0.14	0.08	0.05	0.02
1.5	0.73	0.63	0.45	0.27	0.13	0.08

The toxicity of flumioxazin to *Chironomus riparius* was based on the initial measured concentrations in test sediment. Emergence of *C. riparius* was first observed on day 11. By day 23, all vessels had 100 % emergence (20 out of 20) of *C. riparius*. There was no significant reduction in emergence in any of the treatments relative to the solvent control group. The highest measured concentrations resulting in no observable reduction of emergence, hence survival of *C. riparius* after 23 days exposure (NOEC) was 0.73 mg/kg in the sediment.

RMS Comment (DAR 1997)

Study conducted in compliance with GLP. The study is valid. This study demonstrates that the normal use of flumioxazin will not present a significant risk to sediment dwelling insects.

RMS Comment (2012)

The reported study is GLP compliant and and was conducted according to ASTM guideline (1995), which is in line with the current OECD 218 guideline (2004). Natural sediment was used in the study instead of formulated sediment preferred by the OECD guideline, which is also acceptable. The test results are in compliance with the current guideline's validity criteria (the emergence in the control at least 70% at the end of the test; emergence to adults in control should occur between 12 and 23 days; oxygen concentration should be at least 60% of air saturation value; pH of overlying water should be in the 6-9 range; the water temperature should not differ by more than ± 1.0 ºC). The study is acceptable for regulatory use.

5.4.5 COMPARISON WITH CRITERIA FOR ENVIRONMENTAL HAZARDS (SECTIONS 5.1 – 5.4)

Degradation

RAC agrees with the DS proposal to consider flumioxazin as not rapidly degradable. The substance is demonstrated to be not readily biodegradable and to be not ultimately degraded to a level greater than 70% in water simulation test.

Bioaccumulation

Based on experimental data flumioxazin has a $\log K_{ow}$ of 2.55. No measured bioaccumulation data are available. The measured $\log K_{ow}$ is below the decisive CLP criterion ($\log K_{ow}>4$). In addition, the calculated $\log K_{ow}$ values for the major metabolites are below 1.

Aquatic toxicity

Acute aquatic hazard

Acute toxicity data were available for all three trophic levels. The most sensitive aquatic species is *Lemna gibba.* The lowest reliable short-term aquatic toxicity result is 14d EC_{50}=0.00035mg/L (initial measured concentration).

Chronic aquatic hazard

Reliable and relevant long-term aquatic toxicity data were available for all three trophic levels. The lowest value is for *Navicula pelliculosa,* with a 5d EC_5=0.000041 mg/L (initial measured concentration).

RAC concluded that the key study should be *Lemna gibba*, which results in a NOEC=0.000051 mg/L (initial measured concentration). Due to the hydrolytic unstable conditions of the substance a semi-static test is preferable instead of a static one (*Navicula pelliculosa*). In any case, both the studies determine the same classification and the same M-factor.

Conclusion on classification

Flumioxazin is considered not readily and rapidly degradable and does not fulfil the criteria for bioaccumulation. The lowest acute toxicity value falls within the range $0.0001 < L(E)C_{50} \leq 0.001$ mg/L and the lowest chronic toxicity value lies in the toxicity range of $0.00001 < NOEC \leq 0.0001$ mg/L.
RAC concludes that flumioxazin fulfils the CLP criteria for classification as **Aquatic Acute 1** with an **M-factor of 1000** and **Aquatic Chronic 1** with an **M-factor of 1000**.

Conclusion of environmental classification according to Regulation EC 1272/2008:

Pictogram: GHS09 Signal word: Warning Aquatic acute 1, M = 1000, H400: Very toxic to aquatic life. Aquatic chronic 1, M = 1000, H410: Very toxic to aquatic life with long lasting effects.

Justification for the proposal:

Aquatic acute category 1 (H400) follows from the acute toxicity of the active substance to *Lemna gibba*: $EC_{50} < 1$ mg a.s./L (EC_{50} = 0.00035 mg a.s./L, Hoberg, 1996b). A M-factor of 1000 is applicable based on $0.0001 < LC_{50} \leq 0.001$ mg a.s./l.

Aquatic chronic category 1 (H410) follows from the chronic toxicity of the active substance to *Navicula pelliculosa*: NOEC ≤ 1 mg a.s./L (NOEC < 0.000042 mg/L, Hoberg, 1996a) and the fact that the active substance is not readily biodegradable and not rapidly biodegradable. A M-factor of 1000 is applicable based on $0.00001 < NOEC \leq 0.0001$ mg/l.

Pictogram is required for 'Aquatic acute 1' and 'Aquatic chronic 1' category substance.

Signal word 'Warning' is required for 'Aquatic acute 1' and 'Aquatic chronic 1' category substance.

The statements P273, P391 and P501 are required for 'Aquatic acute 1' and 'Aquatic chronic 1' category substance.

Conclusion of environmental classification and labelling according to Directive 67/548/EEC:

N Dangerous for the environment. R50 Very toxic to aquatic organisms. R53 May cause long term effects in the environment. S60 This material and its container must be disposed of as hazardous waste. S61 Avoid release to the environment. Refer to special instructions/Safety Data Sheet.

Justification for the proposal:

R50 follows from the acute toxicity of the active substance to the most sensitive tested aquatic organisms with EC_{50} < 1 mg a.s./L (*Lemna gibba*: EC_{50} = 0.00035 mg a.s./L, Hoberg, 1996b).

R53 follows from the fact that the active substance is not readily biodegradable.

The safety phrases S60 and S61 have to be applied based on the proposed R50/53.

5.4.6 CONCLUSIONS ON CLASSIFICATION AND LABELLING FOR ENVIRONMENTAL HAZARDS (SECTIONS 5.1 – 5.4)

Conclusion of environmental classification according to Regulation EC 1272/2008:

Pictogram

Signal word	**Warning**
Classification categories:	**Aquatic acute 1**
	Aquatic chronic 1
M-factor (acute/chronic)	**1000/1000**
Hazard statements	**H400: Very toxic to aquatic life.**
	H410: Very toxic to aquatic life with long lasting effects.
Precautionary statements	**P273 Avoid release to the environment**
	P391 Collect spillage
	P501 Dispose of contents/ container to ... (in accordance with local/ regional/ national/ international regulation (to be specified))

Conclusion of environmental classification and labelling according to Directive 67/548/EEC:

Hazard symbol:		**Dangerous for the environment**
Risk phrases:	**R 50/53**	**Very toxic to aquatic organisms, may cause long term adverse effects in the aquatic environment**

Safety phrases:	**S60**	**This material and its container must be disposed of as hazardous waste**
	S61	**Avoid release to the environment. Refer to special instructions/safety data sheets**

RAC evaluation of environmental hazards

Summary of the Dossier submitter's proposal

Flumioxazin currently has a harmonised classification as Aquatic Acute 1 (M-factor=1000) and Aquatic Chronic 1 according to CLP. The dossier submitter (DS) carried out the environmental hazard assessment in order to determine the chronic M-factor, currently not included in Annex VI of the CLP Regulation, taking into account the new criteria brought in by the 2nd ATP to CLP and which are related to the classification of long-term hazards to the aquatic environment.

Degradation

Two hydrolysis studies according to guideline EPA-FIFRA 161-1 and in compliance with GLP were run at pH 5, 7 and 9 at 25 °C for 30 days. Flumioxazin was rapidly hydrolysed in all three buffered solutions and the degradation rate increased with pH (DT_{50}: 3-5 d at pH=5, 19-26h at pH=7, 14-23min at pH=9). Degradation proceeded via opening of the cycloimide ring at all pH values to form 482-HA (7-fluoro-6-[(2-carboxyl-1-cyclohexenoyl)amino]-4-(2-propynyl)-1,4-benzoxazin-3-(2H)-one). Subsequent cleavage of the amide linkage to form APF (6-amino-7-fluoro-4-(2-propynyl)-1,4-benzoxazin-3-(2H)-one) and THPA (3, 4, 5, 6-tetrahydrophthalic acid) was observed only at pH 7 and 5. A supportive hydrolysis study performed with flumioxazin and its degradation product 482-HA showed that the hydrolysis of flumioxazin proceeds predominantly through neutral and base catalyzed processes, while the hydrolysis of 482-HA proceeds predominantly through an acid catalyzed process. Half-lives of 482-HA were calculated to be 2.35 hours, 10.7 days and 72 days at pH 5, 7 and 8, respectively.

The photodegradation of flumioxazin in water was studied according to guideline EPA-FIFRA 161-2. The two studies, in compliance with GLP, were carried out at 25 °C and pH 5.0 for 30 days. Light slightly enhanced degradation of flumioxazin in water at pH 5 and a different degradation pathway was involved. The DT_{50} of flumioxazin was 21 h in the light, while DT_{50} in the dark was 118h. 482-PHO (N-(2-propynyl)-4-[4-carboxy-3-fluoro-2-(3,4,5,6-tetrahydrophthalimido)-2-butenylidene]azetidine-2-one) and THPA were identified as major photolytic degradation products.

A ready biodegradability test was performed according to OECD guideline 301 B. The study was carried out in compliance with GLP at 22°C for 28 days using as the inoculum an activated sludge not previously knowingly exposed to the test substance. Biodegradation of flumioxazin was 3% at the end of the test on day 28 so the substance is considered not readily biodegradable under the conditions of the test.

A water/sediment simulation study, carried out according to SETAC guideline, using radio-labelled flumioxazin was run for 98 days at 20 °C using two systems (clay loam, 8% OC and sandy clay loam, 3.6% OC). Flumioxazin was temporarily found in sediment (max 27% after 7d) and it rapidly disappeared in both water and sediment phases. For the whole systems, DT_{50}s were < 1.85d and DT_{90} were 25-69d. Degradation occurred via hydrolysis to APF (max 58% in water after 7 d) and THPA (max 63% in water and 18% in sediment after 7d). Non-extractable residues reached averages of 38-61% and mineralisation reached averages of 5-29% after 98 days. The available water sediment study has been reassessed using FOCUS kinetics approach, and as no reliable $DegT_{50}$ values have been obtained for water and sediment, the geometric mean value of 21.6 days was used for the water phase and the default value of 1000 days was used for the sediment phase.

A further water/sediment study (Shibata, 2011) was carried out according to OECD Guideline 308. The study was run for 30d and two systems were set up containing natural sediment and associated water and suitable traps for collecting volatile compounds. The degradation rate of flumioxazin in water in the absence or presence of sediment or light, is largely unaffected, indicating that sediment or light insignificantly

contribute to dissipation/degradation.

In this study all major metabolites formed were identified and the presence of sediment decreased the amounts of all metabolites formed. For the whole system and natural water the maximum levels of CO_2 are about 24% and 14.8% (in illuminated conditions) respectively. Moreover, in illuminated water/sediment systems CO_2 and bound residues reached levels ≥48% after 30 days.

Bioaccumulation

The substance has a measured $logK_{ow}$ of about 2.55 (OECD 107, 20 °C, purity 99.5%). The DS did not provide any studies on bioaccumulation.
With a $logK_{ow}$ < 4 the substance does not meet the criterion for bioaccumulation according to CLP.

The 3 major hydrolytic degradation products: 482-HA, APF and THPA have calculated $logK_{ow}$ values of 0.804, 0.127 and 0.88 respectively. The DS indicated that for these data it isn't necessary to carry out a bioaccumulation study in fish.

Aquatic toxicity:

Several acute and chronic aquatic toxicity data are available from studies on the tested substance which, in the majority, followed guideline standards and were in compliance with GLP and reliable according to the DS.

The available short-term tests for flumioxazin were: three for fish, one with invertebrates, three with algae and aquatic plants, respectively. The most sensitive species tested is the aquatic plant *Lemna gibba* (14d semi-static condition test) with an EC_{50}=0.00035 mg/L based on initial measured concentrations, which ranged from 85 to 90% of the nominal concentrations and decreases by 23% at day 3.

The chronic aquatic toxicity of flumioxazin is assessed on the base of three long-term fish tests, four chronic tests with invertebrates and three studies with algae and aquatic plants. The most sensitive species tested was *Navicula pelliculosa* that was exposed to flumioxazin for 120h in static conditions, with the resulting value for NOEC<0.000042 mg/L and EC_5=0.000041 mg/L based on initial measured concentration.

The key studies results proposes by DS are highlighted in bold in the table below.

Method	**Test organism**	**Test system**	**Results**			**Remarks**	**Reference**
			Endpoint	**LC_{50}/EC_{50} [mg/L]**	**NOEC [mg/L]**		
EPA 72-1	*Oncorhynchus mykiss*	Flow-through 96 h	mortality	2.3	0.92	mm	Takimoto et al., 1989b
EPA 72-1	*Lepomis macrochirus*	Flow-through 96 h	mortality	> 21	3.9	mm	Takimoto *et al.,* 1989a
OECD 204	*Oncorhynchus mykiss*	Flow-through 21 d	weight reduction	> 1.2	0.37	mm	Sword *et al.,* 1992
EPA 72-2	*Daphnia magna*	Flow-through 48 h	immobilization	5.9	8.54	mm	Reed *et al.,* 1992
EPA 72-4	*Daphnia magna*	Flow-through 21 d	reproduction	-	0.057	mm	Drottar *et al.,* 1994

OECD 211	*Daphnia magna*	Semi-static 21 d	growth reduction	-	0.1	nom	Cafarella, 2000
ASTM E 1398-94	*Chironomus riparius*	Static 23 d	emergence, survival	-	0.73 (mg a.s./kg sediment)	im	Mattock D. (1997)
OECD 201	*Pseudokirchne-riella Subcapitata*	Static 72 h	cell number inhibition	0.000852	0.000383	im	Blasberg *et al.,* 1992
EPA 122-2, 123-2	***Navicula pelliculosa***	Static 120 h	cell number inhibition	0.0015	**< 0.000042** EC_5= 0.000041	im	Hoberg, 1996a
EPA 122-2, 123-2	***Lemna gibba***	Semi-static 14 d	biomass reduction	**0.00035**	0.000051	im	Hoberg, 1996b
mm – mean measured concentration im – initial measured concentration nom – nominal concentration							

Comments received during public consultation

Four Member States (MS) contributed during public consultation stating a general agreement with the proposed environmental classification.

Two MS had specific comments. They suggested recalculating the EC_{50} value for *Lemna gibba* using data at day 7 (if available) instead of data at day 14 according to OECD test guideline 221. The DS replied that the 7-day EC_{50} is not available.

Concerning the study with *Navicula pelliculosa,* one MS noted that the EC_{50} and NOEC were measured after 5 days, while in OECD guideline 201 for freshwater algae the exponentially growing test organisms were exposed over a period of 72 hours.

Another MS suggested using the value of EC_5=0.000041 mg/L instead of the NOEC from the study with *Navicula pelliculosa*. In this regard, the DS stated that using the EC_5 would influence neither the classification of the substance nor derivation of a chronic M-factor.

A further MS, while agreeing that *Lemna gibba* and *Navicula pelliculosa* were the most sensitive species, highlighted that the EC_{50} and NOEC values are based on the initial mean measured concentration, while it would have been more appropriate to calculate the geometric mean concentration at the start and the end of the test, taking into account that the substance is hydrolytically unstable. This could have an influence on the setting of the M-factor.

The DS stated that according to SANCO 3268/2001/rev.4, the endpoints based on initial measured concentrations are considered relevant when effect data are obtained from the test performed under static conditions.

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the DS proposal to consider flumioxazin as not rapidly degradable. The substance is demonstrated to be not readily biodegradable and to be not ultimately degraded to a level greater than 70% in water simulation test.

Bioaccumulation

Based on experimental data flumioxazin has a $logK_{ow}$ of 2.55. No measured

bioaccumulation data are available. The measured $logK_{ow}$ is below the decisive CLP criterion ($logK_{ow}$>4). In addition, the calculated $logK_{ow}$ values for the major metabolites are below 1.

Aquatic toxicity

Acute aquatic hazard

Acute toxicity data were available for all three trophic levels. The most sensitive aquatic species is *Lemna gibba.* The lowest reliable short-term aquatic toxicity result is 14d EC_{50}=0.00035mg/L (initial measured concentration).

Chronic aquatic hazard

Reliable and relevant long-term aquatic toxicity data were available for all three trophic levels. The lowest value is for *Navicula pelliculosa,* with a 5d EC_5=0.000041 mg/L (initial measured concentration).

RAC concluded that the key study should be *Lemna gibba*, which results in a NOEC=0.000051 mg/L (initial measured concentration). Due to the hydrolytic unstable conditions of the substance a semi-static test is preferable instead of a static one (*Navicula pelliculosa*). In any case, both the studies determine the same classification and the same M-factor.

Conclusion on classification

Flumioxazin is considered not readily and rapidly degradable and does not fulfil the criteria for bioaccumulation. The lowest acute toxicity value falls within the range 0.0001 < $L(E)C_{50}$ ≤ 0.001 mg/L and the lowest chronic toxicity value lies in the toxicity range of 0.00001 < NOEC ≤ 0.0001 mg/L.
RAC concludes that flumioxazin fulfils the CLP criteria for classification as **Aquatic Acute 1** with an **M-factor of 1000** and **Aquatic Chronic 1** with an **M-factor of 1000**.

Supplemental information - In depth analyses by RAC

Aquatic toxicity

Regarding the semi-static test on *Lemna gibba*, the frequency of renewal of solution, (specific intervals at which it was periodically replaced during the test) was not specified in the CLH report, but it was stated that the initial mean measured concentration decreased to 23% of the nominal concentrations at day 3. For the static test on *Navicula pelliculosa* there was only the initial measured concentration. However the EC_{50} and NOEC of both studies are based on initial measured test concentrations instead of mean measured concentration, as suggested by OECD 221.

6 OTHER INFORMATION

None.

Aquatic toxicity

Regarding the semi-static test on *Lemna gibba*, the frequency of renewal of solution, (specific intervals at which it was periodically replaced during the test) was not specified in the CLH report, but it was stated that the initial mean measured concentration decreased to 23% of the nominal concentrations at day 3. For the static test on *Navicula pelliculosa* there was only the initial measured concentration. However the EC_{50} and NOEC of both studies are based on initial measured test concentrations instead of mean measured concentration, as suggested by OECD 221.

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ABBREVIATIONS

AV	atrioventricular
EDD	embryonic development day
DMSO	dimethyl sulphoxide
GD	gestation day
GLP	good laboratory practice
Hb	haemoglobin
HW	Han Wistar
ICR	imprinting control region
JW	Japanese white
M/E	myeloid / erythroid
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
NaB	sodium butyrate
NZW	New Zealand white
PBPK	physiologically based pharmacokinetic
PCE	polychromatophilic erythroblast
PPO	protoporphyrinogen oxidase
PPIX	protoporphyrin IX
RBC	red blood cell
S-53482	flumioxazin
sd	standard deviation
SD	Sprague Dawley
VSD	ventricular septal defects
WBC	white blood cell
CLP	Regulation (EC) 1272/2008 of the European Parliament on the Classification Labelling & Packaging of substances and mixtures.
mg/kg	milligrams per kilogram
mg/kg/d	milligrams per kilogram per day
ppm	parts per million
NOAEL	no observed adverse effect level
ATP of the DSD	Adaptation to the Technical Progress of the Dangerous Substances Directive (67/548/EEC)
bw	bodyweight

LC/MS	liquid chromatography/ mass spectrometry
GOT	glutamic-oxaloacetic transaminase
DNA	deoxyribonucleic acid
HPLC	High Performance Liquid Chromatography
HRF	human relevance framework
MOA	mode of action
ECHA	European Chemicals Agency
ICPS	International Programme on Chemical Safety

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ANNEXES

ANNEX 1

DISCUSSION ON HUMAN RELEVANCE OF THE DEVELOPMENTAL EFFECTS INDUCED BY FLUMIOXAZIN (BASED ON NEW DATA SINCE THE PUBLICATION OF FLUMIOXAZIN IN THE 28TH ATP OF THE DSD)

SBT-0122

An update of a discussion on human relevance of the developmental effects induced by flumioxazin in rats

Sumitomo Chemical Co., Ltd.

Environmental Health Laboratory

2012. 5. 31

Prepared by Satoshi Kawamura Ph. D.

An update of a discussion on human relevance of the developmental effects induced by flumioxazin in rats

Executive summary

Flumioxazin caused embryolethality, teratogenicity [mainly ventricular septal defects and wavy ribs], and growth retardation in rats at 30 mg/kg without maternal toxicity but not in rabbits at the maternal toxicity level of 3000 mg/kg. Flumioxazin was classified as category 2 R61 or category 1B (CLP) for developmental toxicity due to the rat developmental effects and presumed relevance to humans.

Sumitomo Chemical Company has carried out an extensive program of research with flumioxazin and has successfully elucidated the mechanism of the developmental toxicity in rats. Existing mechanistic studies, which were evaluated during the previous review of flumioxazin for Annex 1 inclusion, have been supplemented by additional studies to strengthen the mechanistic case and to allow an assessment of the relevance to human of developmental effects found in rats.

There is convincing evidence for a single mode of action causing the developmental toxicities in the rat. The sequence of key biological events in the proposed mode of action has been elucidated. Inhibition of PPO interferes with normal heme synthesis, which causes loss of blood cells leading to fetal anemia, embryolethality and the development of malformations.

Rats are particularly sensitive to the effects of protoporphyrinogen oxidase (PPO) inhibition induced by flumioxazin in erythroblasts. This leads to anemia that is a critical precursor of the developmental toxicity resulting from flumioxazin exposure. In contrast, humans are unlikely to develop anemia from PPO inhibition. This conclusion is based on (1) clinical findings that PPO deficient patients with Variegate Porphyria show no signs of anemia, (2) experimental evidence that flumioxazin does not reduce heme production in K562 cells, which are derived from human erythroleukemia, and (3) that humans are less sensitive to PPO inhibition than rats.

Pharmacokinetic modeling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at exposure equivalent to a maternal dose exceeding 1000 mg/kg/day, thus demonstrating the large species difference in sensitivity.

Overall, it is concluded that the rat is an inappropriate model for assessing the developmental toxicity of flumioxazin in humans because, unlike humans, they are

highly sensitive to PPO inhibition, resulting in fetal anemia and consequent developmental toxicity. There is considered to be no plausible scenario whereby humans would be at risk of developmental toxicity given the species differences in susceptibility to flumioxazin and potential for anemia.

Contents

1. Introduction

Flumioxazin is an N-phenylimide herbicide. The herbicidal activity of flumioxazin is due to photodynamic action of accumulated protoporphyrin IX (PPIX) resulting from inhibition of protoporphyrinogen oxidase (PPO), which is an enzyme in porphyrin biosynthesis and common to plants and animals as part of chlorophyll and heme biosynthesis (Fig. 1). In mammals eight enzymes are involved in the heme biosynthetic pathway which starts in the mitochondria and, after passing through cytoplasmic stages, re-enters the mitochondria for the final steps of heme formation. The first and last three enzymes including PPO are located in mitochondria while the others are in the cytosol. It is postulated that the mechanism of PPIX accumulation *in vivo* resulting from PPO inhibition is as follows: the accumulating protoporphyrinogen eventually leaves the mitochondria, enters the plasma, and is oxidized nonenzymatically there to PPIX. Because of abnormal subcellular location, the resulting PPIX is beyond reach of ferrochelatase and cannot be transformed to heme (1). In humans malfunction of this pathway leads to metabolic disorders termed porphyria.

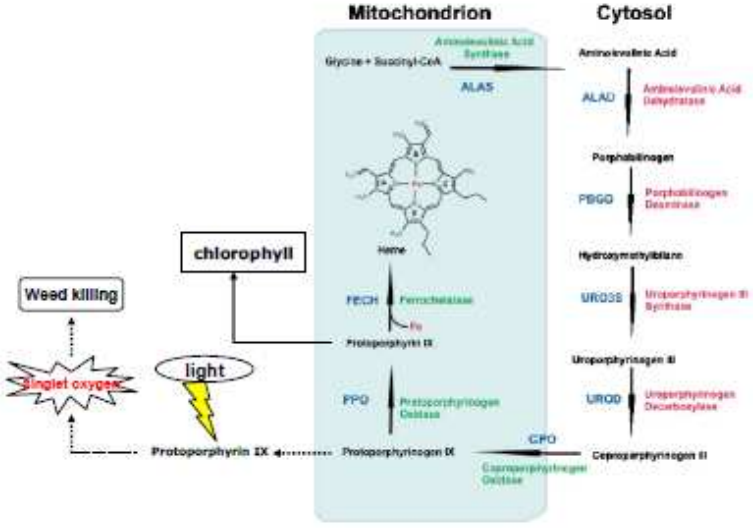

Fig. 1 Heme biosynthetic pathway and mode of herbicidal action of flumioxazin

In studies, there were striking species-specific differences in developmental toxicity resulting from flumioxazin exposure. Flumioxazin produced embryolethality, teratogenicity [mainly ventricular septal defects (VSD) and wavy ribs], and growth retardation in rats exposed to 30 mg/kg without maternal toxicity. In contrast, these

effects were not seen in rabbits exposed to doses as high as the maternal toxic level of 3000 mg/kg. Flumioxazin has been classified as category 2 R61 or category 1B (CLP) for developmental toxicity due to the rat developmental effects (Annex VI of Regulation (EC) No. 1272/2008).

Sumitomo Chemical Company has carried out an extensive program of research with flumioxazin, enabling them to elucidate the mechanism of developmental toxicity in rats. Additionally, they have assessed the relevance of these findings to predictions of developmental toxicity in humans. This research has demonstrated that developmental toxicity in rats is due primarily to PPO inhibition in the fetus, resulting in fetal anemia. This, in turn, leads to the developmental manifestations caused by flumioxazin. Significantly, humans are unlikely to develop anemia associated with PPO inhibition. As a result, the rat is particularly sensitive with respect to the effects of PPO inhibition and thus is an inappropriate model for predicting the potential for developmental toxicity of flumioxazin in humans.

This report summarizes the mechanism for the teratogenicity in rats from the previous review for inclusion of flumioxazin in Annex 1 and discusses the results of subsequent additional studies included in section IIA 5.5.13 of the present dossier and the CLH report.

2. Results of the rat and rabbit teratogenicity studies

In a rat teratology study flumioxazin was administered to dams by gavage during gestational days 6 through 15. Fetuses were removed by Caesarian section and examined for external, skeletal and visceral abnormalities on gestational day 20 (SBT-00-0012). Flumioxazin caused embryolethality, teratogenicity (mainly VSD and wavy ribs), and growth retardation at 30 mg/kg (Table 1). In contrast, flumioxazin caused no developmental toxicity at doses to 3000 mg/kg in rabbits treated during days 6 through 18 of gestation (SBT-11-0017).

Table 1 Summary results of the rat teratology study with flumioxazin

	0 mg/kg	30 mg/kg
Embryonic deaths	5.6%	20.4%
Fetal bodyweight	Male;3.51g Female;3.34g	Male;3.00g Female;2.79g
VSD	1.4%	25.5%
Wavy ribs	0.6%	24.3%

In the rat multigeneration study there was an increase in resorptions and a decrease in both pup survival and average pup weight which were probably seen as an extension of the causal effects which produced embryolethality and growth retardation observed in the rat teratology study (SBT-21-0035)

3. Mechanism of rat teratogenicity induced by flumioxazin

The mechanism of the developmental toxicity of flumioxazin in rats is presented in Fig. 2:

a) Flumioxazin inhibits PPO, which is the penultimate enzyme in heme biosynthesis and is localized in mitochondria (Fig. 1). Its inhibition results in degeneration of fetal erythroblasts leading to anemia.

b) Severe fetal anemia leads to the fetal death.

c) Surviving fetuses are growth-retarded as indicated by a decrease in body weight. They compensate for this anemia by pumping a greater volume of blood which leads to observed enlargement of the heart just prior to closure of the interventricular foramen. This results in delayed closure of the foramen represented as VSD in the term fetus due to mechanical distortion of the heart or abnormal blood flow.

d) Concurrently serum protein is decreased in the fetus resulting in wavy ribs.

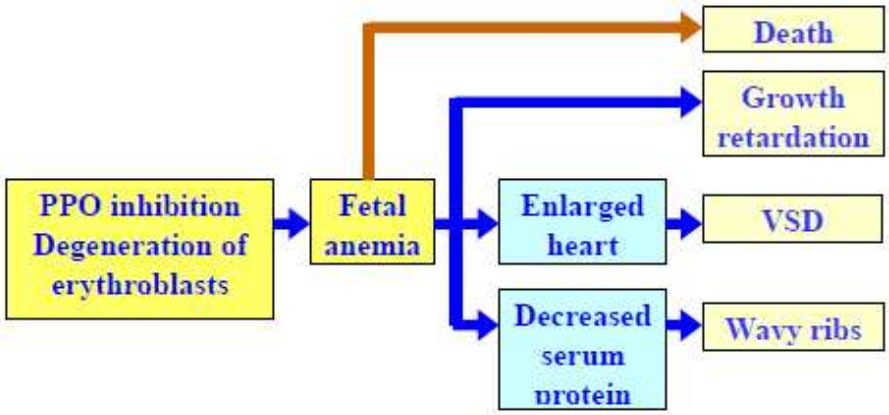

Fig. 2 Mechanism of developmental toxicity induced by flumioxazin

The mechanism described above was based on a series of studies designed to elucidate the bases of the species specific developmental toxicity produced by flumioxazin in rats but not in rabbits. These studies were evaluated during the previous review of flumioxazin for Annex 1 inclusion and were summarized in the DAR. The mode of action described below was endorsed by the Scientific Committee on Plants (Opinion SCP/FLUMIO/002-Final 23 May 2001).

As summary it has been demonstrated:

3.1 The critical period for sensitivity to the developmental effects of flumioxazin including fetal death, reduced fetal bodyweight and VSD is day 12 of gestation (SBT-30-0044). This suggests a common mechanism for the three types of developmental effects.

3.2 A strong correlation exists between PPIX accumulation, considered to result from PPO inhibition, and developmental toxicity. Evidence for this correlation is based on differences between rats and rabbits, the critical period of sensitivity to developmental effects in rats, and compound-specific differences between two chemicals of the N-phenylimide family. The latter are structurally related to flumioxazin: one (S-23121) that produces developmental effects in rats and one (S-23031) that does not (PPT-00-0023, SAT-11-0024). Protoporphyrin IX accumulates in rat fetuses in response to flumioxazin but not in rabbit fetuses (SBT-0061). Protoporphyrin IX accumulation is observed when rat fetuses are treated with the developmentally toxic compounds (flumioxazin and S-23121) that cause significant PPO inhibition (SBT-0062). The peak period of PPIX accumulation in rat fetuses corresponds to its developmental effects (SBT-0063, SBT-30-0044). This correlation demonstrates a close link between PPO inhibition and developmental abnormality.

3.3 Histological examination of rat fetuses at light and electron microscopic levels after oral administration of flumioxazin (1000 mg/kg) to dams on gestational day 12 (the day of greatest sensitivity) demonstrated mitochondrial lesions. These included abnormal iron deposits, probably due to inhibition of heme biosynthesis, in polychromatophilic erythroblasts that were observed as early as 6 hours after treatment. Subsequent degeneration of these erythroblasts was indicative of fetal anemia (SBT-0064).

Histological examination of hearts from exposed embryos revealed thinning of the

ventricular wall by 36 hours after treatment. This may reflect compensation for the loss of embryonic blood cells. Therefore, the VSD caused by flumioxazin appears to result from inhibition of heme biosynthesis rather than from direct injury to embryonic heart tissue.

No effects were observed in rabbits treated in the same manner as rats (SBT-0064). The observed difference in histological changes between rats and rabbits completely corresponds to those of developmental toxicity and PPIX accumulation caused by exposure to flumioxazin.

3.4 Observations in the pathogenesis of developmental effects of flumioxazin in rat fetuses included: anemia, reduced serum protein, enlarged heart, edema, delayed closure of the interventricular foramen, and incomplete/delayed ossification of the ribs (Figs. 3 and 4).

Severe fetal anemia is observed up to gestational day 16 following treatment with flumioxazin (400 mg/kg) on gestational day 12. Fetal death occurs by gestational day 15 as additional lethality is not observed from gestational day 15 through 20.

Enlarged heart is seen among surviving fetuses in concurrence with severe fetal anemia. This suggests that enlarged heart results from pumping greater volumes of blood in compensation for fetal anemia. Enlargement of the heart precedes interventricular foramen closure. Therefore, the VSD caused by flumioxazin is due to failure of heart closure resulting from mechanical distortion of the heart or abnormal blood flow rather than from direct toxic effects of flumioxazin on cardiac tissue.

Concurrently, decreased serum protein is observed in the fetus, presumably due to reduced production in the liver in response to hypoxia. The resulting osmotic imbalance causes edema. Reduction of fetal serum protein leads to incomplete/delayed ossification of the ribs and the wavy ribs seen at term (SBT-0065).

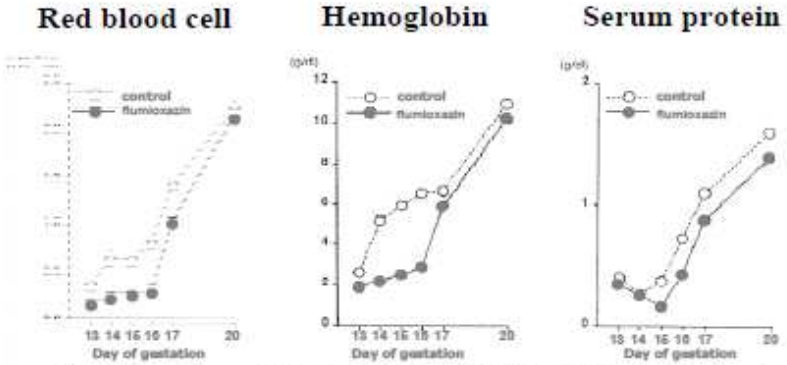

Fig. 3 Hematology and blood chemistry of fetal blood following flumioxazin treatment on gestational day 12

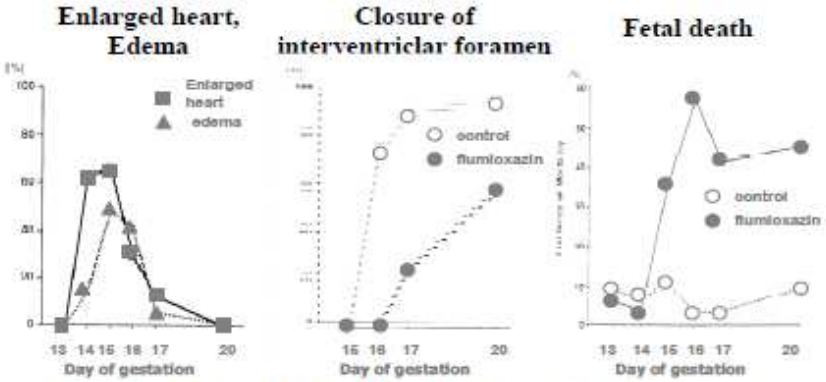

Fig. 4 Morphological observation of fetuses following flumioxazin treatment on gestational day 12

3.5 Species- and compound-related differences were observed in *in vitro* inhibition of PPO and *in vitro* inhibition corresponded closely with PPIX accumulation and teratogenicity. Thus, PPO inhibition is considered to be the primary cause of developmental toxicity in rats. Sensitivity of PPO activity extracted from adult female liver was found to be comparable to that of embryonic PPO, suggesting that inhibition

of adult liver PPO is indicative of embryonic PPO inhibition. Based on the relative sensitivity to inhibition of adult liver PPO in the three species tested (rat>human>rabbit), risk assessments using the NOAEL for studies in the rat protect humans more than adequately (SBT-31-0045, SBT-0060).

4. Relative sensitivity of adult and fetus

Porphyria and sideroblastic anemia were observed in adult rats in studies of the effects of dietary administration of flumioxazin. In sideroblastic anemia, iron cannot be incorporated into hemoglobin and deposits in mitochondria. The initial histological change observed in rat embryos was iron deposits in mitochondria, and PPIX was accumulated in treated embryos in the mechanistic studies with flumioxazin. Thus, rats develop an anemia both in fetuses and adults in the same manner.

Anemia was evident at 1000 ppm (65 - 73 mg/kg/d) and slight anemia was present in females at 300 ppm (22 mg/kg/d) in a rat 13-week subacute study by dietary administration (SBT-10-0023). Anemia was present in the absence of overt toxicity as shown by no adverse clinical signs or body weight and food consumption changes. The developmental LOAEL was 30 mg/kg in the absence of overt maternal toxicity in the rat teratology study by gavage and there is likely to have been an underlying anemia based on the results of the 13-week study. Sensitivity to inhibition of PPO extracted from adult female liver is comparable with that of PPO from embryos, as shown in table 2.

Although direct comparisons between adult and fetal responses to flumioxazin are complicated by differences in routes and duration of exposure, it does not appear that there is a significant difference in sensitivity to the development of anemia between adult and fetal rats.

Table 2 Comparison of IC_{50s} (nM) for rat PPOs derived from adult liver and embryo

Chemicals	S-23031 a)	S-23121 a)	flumioxazin
Adult liver	793	11	7
Embryo day12	344	47	12
Embryo day15	204	20	6

a) The N-phenylimide family structurally related to flumioxazin
S-23031 is non-teratogenic and S-23121 is teratogenic.

5. Evaluation of other potential Mode of Action

5.1 Accumulated PPIX

Because PPIX accumulation corresponded to the developmental toxicity of flumioxazin, it might be assumed that developmental toxicity of flumioxazin is mediated through the same mode of action as the herbicidal one. If photodynamic action is a cause of developmental toxicity, a photodynamic dye should be a developmental toxicant. However a photodynamic dye, rose bengal, exhibited neither embryolethality nor teratogenicity in rats (2). The light would not sufficiently reach embryos through the maternal body wall to induce photodynamic action of accumulated PPIX in embryos.

Protoporphyrin IX is assumed to be an endogenous ligand to the peripheral benzodiazepine receptor on mitochondria. Presumably acting through the receptor, PPIX suppressed DNA replication in mouse spleen lymphocytes *in vitro* (3). Nevertheless many of the benzodiazepoines such as diazepam, lorazepam, clonazepam, and oxazepam failed to exhibit teratogenicity in rats (4). Accumulated PPIX is considered to be indicative of PPO inhibition rather than a causative factor in teratogenicity.

5.2 Form of anemia

Flumioxazin caused hypochromic microcytic anemia that generally occurs as a consequence of impaired hemoglobin synthesis (5). Impairment of hemoglobin synthesis is caused by iron-deficiency or defective porphyrin metabolism resulting in abnormal iron accumulation in erythroblasts termed sideroblasts. As indicated in mechanistic studies, iron deposition in mitochondria was an initial histological change and increased sideroblasts were observed. Flumioxazin induced anemia is due to inhibition of porphyrin metabolism rather than iron deficiency.

5.3 Relationship between fetal death and malformation

In some cases fetal death is attributable to malformation. Beck and Lloyde investigated the relationship between fetal deaths and fetal malformation by treating rats with trypan blue at day 8.5 and examining the uteri and fetuses on days 11.5, 14.5 and 20.5 of gestation (6). Because the incidence of fetal malformations fell with a corresponding rise in fetal deaths as pregnancy proceeded, the authors concluded that fetal death was a result of pre-existing fetal malformation in the majority of cases. In the flumioxazin studies, most of the dead fetuses were observed by day 15 while VSD can be diagnosed following completion of closure of the interventricular foramen on day

16. Consequently, fetuses were dead prior to closure of the interventricular septum indicating that VSD is not the direct cause of conceptal death but rather occurs in some surviving fetuses. This supports fetal anemia as the cause of embryonic deaths rather than deaths from malformations.

5.4 VSD

Initial histological changes observed in rat embryos were iron deposits in mitochondria and dilatation of mitochondrial matrix space in polychromatophilic erythroblasts. Following the mitochondrial lesions, affected erythroblasts degenerated in the embryonic circulation and were engulfed by macrophages. Treatment-related changes in the embryonic cardiovascular system and liver accompanied the appearance of erythroblastic lesions. Thinning of the ventricular walls of the heart is indicative of dilatation of the ventricles, and reflects a compensatory reaction to embryonic anemia since enlargement of heart was observed corresponding to decreased hemoglobin content and reduced red blood cell number. Increased stroke volume in the heart is an important reaction to anemia (7). No treatment-related changes in myocardial cells were observed at the electron microscopic level. As noted previously, the interventricular foramen closes from day 15 to day 16. In our studies, exposed hearts were enlarged from day 14 and completion of ventricular septa formation was delayed.

Clark has proposed five pathogenic modes of actions for some congenital cardiac malformations based on mechanism rather than anatomic anomaly. They are ectomesencymal tissue migration abnormalities, abnormal intracardiac blood flow (cardiac hemodynamics), cell death, extracellular matrix, and abnormal targeted growth (8). Clark stated that perimembranous ventricular septal defect may represent abnormal fusion of the muscular, inflow and outflow septa, and that deviation of the septal components by abnormal blood flow pattern may lead to defects in this region of the heart (9).

A comparison of sensitive periods for development of VSD between flumioxazin and several other agents shows considerable differences in peak sensitivity. X-ray irradiation (10) and nimustine (11), an alkylating agent, or bisdiamnie (12), which acts on the proliferation or migration of mesencyme, probably produce VSD by direct injurious effects (cell damage) on the fetal heart. The peak of sensitivity to these agents occurs between days 8 – 10, while the most sensitive day for flumioxazin-induced-VSD is gestational day 12.

Earlier studies by Haring (13) and Clemmer and Telford (14) support the proposed mechanism by showing that prenatal hypoxia produces cardiovascular abnormalities

including VSD in rats. Jaffee stated that, when hypoxia was used as a teratogenic agent following the onset of circulation, distortion of the form of the heart tube was a primary lesion (15).

Thus, overall it is concluded that VSD caused by flumioxazin is attributed to fetal anemia and not to any other direct injurious effect on the heart.

5.5 Wavy ribs

Wavy ribs are induced in the later stages of rib chondrification and ossification, and may be indicative fetal pathology as opposed to malformations. It may be possible that many agents produce wavy ribs through several mechanisms leading to two final common effects including inhibition of mineralization and increased uterine tone. Renal loop diuretics and beta-stimulants have been studied in detail because they are associated with a high incidence of wavy ribs. Maternal serum chloride was decreased after treatment with furosemide, a renal loop diuretics. Co-administration of a muscular relaxant reduced the incidence of wavy ribs after furosemide exposure. Decreased fetal serum alkaline phosphatase and total protein were reported following exposure of fenoterol, a beta-stimulant (16). Flumioxazin decreased fetal serum protein and increased incomplete/delayed ossification of the ribs. The increased incidence of wavy ribs is more likely to be associated with these changes rather than being caused by a different mechanism.

5.6 Link between fetal anemia and developmental toxicity

A link between fetal anemia and developmental effects observed in the flumioxazin teratogenicity study is also demonstrated in recent studies with artesunate, an anti-malarial drug. Artesunate induces developmental abnormalities consisting of fetal death, growth retardation and anomalies such as VSD, rib abnormalities and bent long bones (17). Embryonic erythroblasts are the primary target of artesunate toxicity and consequent embryonic anemia resulted in developmental toxicity similar to that produced by flumioxazin (18).

5.7 Species difference in metabolism between rats and rabbits

When pregnant rats and rabbits received oral administration of ^{14}C-flumioxazin at 30 mg/kg for seven consecutive days, no clear pattern of absorption, distribution, metabolism or excretion was seen that could account for the species specific developmental toxicity in rats. After initial dose, Cmax/min of ^{14}C concentration in plasma ranged from 4.49 to 0.70 in rats and from 4.14 to 1.02 in rabbits. In both species

most of the previous dose of ^{14}C was excreted before the next dose, and the metabolic profiles of flumioxazin were similar (19).

5.8 PPO inhibitory activity of metabolites

Oral doses of [phenyl-^{14}C] flumioxazin (30 mg/kg) administered to pregnant rats from gestational days 6 through 12 cross the placenta and reach the rat fetus. Major metabolites in the fetus included 3OH-flumioxazin, 4OH-flumioxazin, and APF (19). In order to determine the active form that inhibits PPO, we employed *in vitro* PPO inhibition assays using liver extracts prepared from adult, female livers. Experiments were conducted with flumioxazin and its three major metabolites (20). The results showed that flumioxazin was the strongest PPO inhibitor. There was no metabolite that could account for the species-specific developmental toxicity in rats based on the degree of PPO inhibition.

The possibility of a direct effect of metabolites on developmental toxicity is also considered. APF was detected at higher concentrations in rat fetuses compared with other metabolites. It is a benzoxazinone moiety formed from cleavage of the amide linkage. There is convincing evidence that embryolethality and VSD are attributed to the consequences of fetal anemia and that the fetal malformations are not the causative factors in embryonic deaths. The spectrum of developmental effects associated with flumioxazin is consistent with a single mode of action. Therefore, it is considered very unlikely that a metabolite would be a direct acting teratogen causing VSD and skeletal anomalies by a mechanism that was independent of fetal anemia and embryolethality. Furthermore, the main metabolite APF was also detected in rabbit fetuses and no developmental toxicity was seen in rabbits at a dose level 100 times higher than that causing developmental toxicity in the rat. Fetal concentrations of metabolites at this dose level in the rabbit would be much higher than those in the rat.

A peak sensitive period was common to embryolethality, teratogenicity and growth retardation with flumioxazin and the effect levels were identical at 30 mg/kg in successive administrations and at 400 mg/kg in single doses during the sensitive period. The evidence supports a single mode of action to be the basis of the three types of developmental toxicities.

In conclusion there is no compelling evidence for any other MOA for the developmental toxicity of flumioxazin in the rat.

6. Critical biological events for the rat teratogenicity and their relevance to humans

As has been elucidated, PPO inhibition by flumioxazin is the primary cause of a sequence of developmental effects on the rat fetus, and anemia is the critical event resulting in developmental toxicities including morphological abnormalities.

The initial site of hemopoiesis in the embryo is the yolk sac, later shifting to the liver. Erythroblasts derived from yolk sac are known to actively synthesize hemoglobin at mid-gestation. The initial changes observed in flumioxazin-exposed rat embryos were iron deposits in mitochondria and dilatation of mitochondrial matrix space in polychromatophylic erythroblasts, which are derived from yolk sac. The accumulation of iron destined for incorporation into porphyrin would be provoked by deficient heme biosynthesis in mitochondria in erythroblasts (Fig. 1). Polychromatophilic erythroblasts are intermediate-stage erythroblasts in erythroid maturation that actively synthesize hemoglobin. Late erythroblasts, which are called orthochromatophilic erythroblasts, are postmitotic. A characteristic of yolk sac hemopoiesis is that erythroid cells synchronously undergo maturation as a relatively homogeneous population. Thus cell death caused by flumioxazin induces an enormous loss of blood cells in developing rat embryos, presumably because most erythroid cells are lost synchronously and remaining cells no longer proliferate. As noted, a link between fetal anemia and developmental effects has also been demonstrated in studies with artesunate, which induces fetal anemia and developmental toxicity similar to that produced by flumioxazin.

Overall, it is concluded that the postulated mechanism of developmental toxicity in the rats has been elucidated with a high degree of confidence. Sumitomo has further investigated the following biological events in order to assess their relevance as indicators of potential developmental toxicity of flumioxazin in humans. The new studies referred to in this section are summarized in IIA 5.6.13 of the dossier and the CLH report.

6.1 Relative sensitivity to inhibition of PPO by flumioxazin among species

Since PPO inhibition is the initial events in the developmental toxicity of flumioxazin, species differences in PPO inhibition *in vitro* (liver) in rats and humans were investigated as part of the mechanistic studies described above. Inhibition curves are shown in Fig. 5. Rat PPO was most sensitive to inhibition by flumioxazin among the species tested while humans were intermediate between rats and rabbits (Table 3).

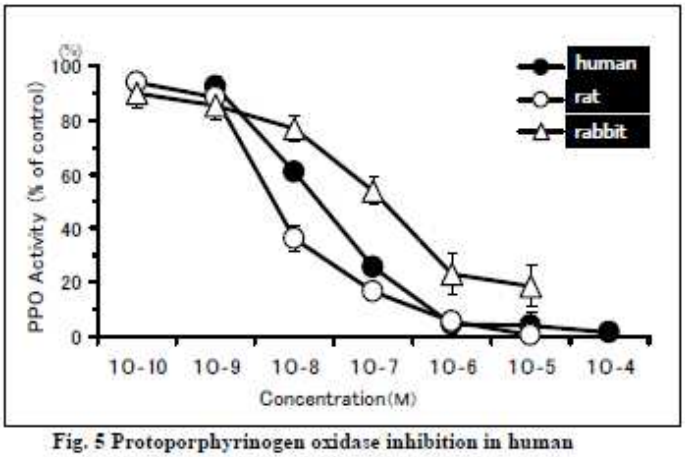

Fig. 5 Protoporphyrinogen oxidase inhibition in human

Table 3 Comparison of IC_{50}s (nM) among three species for PPOs derived from adult liver

Species	rat	rabbit	human
IC_{50}	7.15	138	17.3

PPO exists in complicated and highly-organized structure in mitochondria. Accessibility of flumioxazin to PPO in the cell can be different from that in the *in vitro* model employing mitochondrial extracts. Therefore, PPO inhibition by flumioxazin was investigated using cryopreserved hepatocytes of rats, rabbits, monkeys and humans (21). The results are shown in Fig. 6. No PPIX accumulation was observed in rabbit and monkey hepatocytes at the maximum tested concentration of flumioxazin. Remarkable accumulation of PPIX as the result of PPO inhibition in hepatocytes was observed in rats with a smaller amount of PPIX detected in human hepatocytes. These results show that human PPO is less sensitive to flumioxazin than rat PPO at the cellular level.

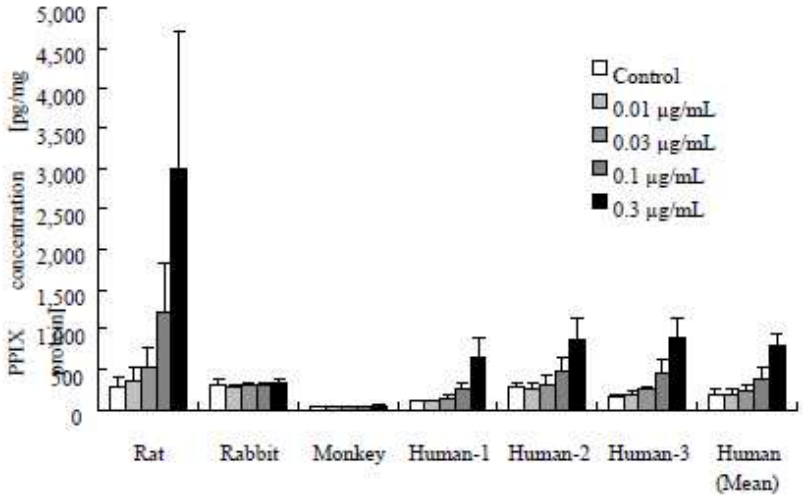

Fig. 6 PPIX accumulation in cryopreserved hepatocytes induced by flumioxazin

6.2 Induction of anemia by PPO inhibition

In general toxicity studies and teratogenicity studies of flumioxazin, the rat is the most sensitive animal species among rats, mice, dogs and rabbits. Anemia, attributable to PPO inhibition, is the primary toxic effect in rats caused both in adults and embryos.

Sumitomo has investigated whether or not PPO inhibition in erythroblasts can cause anemia in humans. Porphyrias are disorders in which the activities of the enzymes of the heme biosynthetic pathway, including PPO, are deficient. They can be classified as either hepatic or erythropoietic, depending on the principal site of expression of the specific enzymatic defect. The tissue-specific expression of porphyrias is largely due to the tissue-specific control of heme pathway gene expression, especially at the level of aminolevulinate synthase (ALAS), the first and rate-limiting enzyme of heme biosynthesis (Fig.1) (22). In liver, hemoprotein enzymes are rapidly turned over in response to current metabolic needs. The activity of ALAS1, the housekeeping isoenzyme of ALAS, in normal liver is the lowest among all enzymes in the heme biosynthetic pathway. In erythroid cells, the activity of ALAS2 (the erythroid-specific isoenzyme of ALAS) is induced only during the period of active heme synthesis, and is regulated by the amount of free iron present (23).

Variegate porphyria (VP) is a disease associated with PPO deficiency. VP is categorized

as hepatic porphyria and the main symptoms are neuronal manifestation and dermal inflammation. Hepatic porphyrias usually do not include anemia or hematological problems. Anemia was not found in hepatic porphyrias attributable to marked deficiency of delta aminolevulinic acid dehydratase (ALAD), coproporphyrinogen oxidase (CPO), or PPO. This suggests that defective enzymatic activity resulting in disturbances in heme biosynthesis in liver does not necessarily limit heme synthesis in erythroid cells (22). Variegate porphyria is associated with reduced PPO content and ALAD activity in erythrocytes. Erythrocytes counts were not affected by VP and hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin in VP were slightly higher than their controls. The low rate of heme production in VP is enough to generate the same, or even greater, quantity of hemoglobin as control women (24).

In contrast to VP, erythropoietic protoporphyria (EPP) resulting from deficiency of ferrochelatase (FECH), the last enzyme in the heme biosynthetic pathway (Fig.1), sometimes includes mild anemia with hypochromia and microcytosis or mild anemia with reticulocytosis. Iron accumulation in erythroblasts and ring sideroblasts occur in some EPP patients (22). Microcytic anemia occurs in 20% to 60% of patients. Erythropoiesis was impaired in most patients with dominant EPP from the UK and France and all had a downward shift in hemoglobin (25). FECH deficiency in EPP results in the accumulation of protoporphyrin almost exclusively in erythroid tissue, even though FECH is deficient in all other tissues in these patients. This finding suggests that FECH activity can become rate limiting in erythroid cells, but not in other tissues when the enzyme itself or its substrate, iron, is partially deficient (23).

Recently families with X-linked, dominant protoporphyria (XLDPP) have been described. Patients with this disorder have normal FECH activities, indicating that protoporphyrin accumulation is not caused by FECH deficiency (25). Patients showed neither anemia nor iron overload. Disruption of the C-terminal region of ALAS2 leads to markedly increased ALAS2 activity and the production of protoporphyrin in excess of the amount required for hemoglobinization. These findings suggest that the rate of ALA formation is increased to such an extent that insertion of Fe into PP by FECH becomes rate limiting for heme synthesis (26).

These clinical findings demonstrate that PPO activity in human erythroid cells is much higher than FECH or ALAS2 activity, which is rate-limiting in heme biosynthetic pathway in human erythroids. It is therefore unlikely that PPO deficiency would induce anemia or disturbances of heme synthesis in human erythroid cells. In contrast, the results of toxicity studies in rats suggest that in rat erythroid cells, PPO activity is close

to a rate-limiting enzyme activity. Therefore decreased PPO activity becomes rate-limiting in porphyrin production in erythroids resulting in PPIX accumulation, iron deposit, and anemia.

Enzymatic activities from various tissues are presented in Table 4 (27-32). Although the data are derived from non-erythroid tissues, we present them to illustrate relative activities in human and rat tissues. In humans PPO activity could be higher than other enzymes. In rats FECH activity varies and PPO activity is not necessarily higher than FECH.

Table 4 Activities of enzymes in the heme synthetic pathway (nmol/h/mg protein)

		ALAS	CPO	PPO	FECH
Human	Fibroblast	0.003 - 0.005	-	2.12	0.032
	Liver	-	0.6	10.8	3.72
	Leucocyte	-	-	8.73	0.24
rat	Liver (mitochondria)	-	-	3	87
	Liver (homogenate)	-	1.2	10.2	3.18 - 4.2
	Liver (mitochondria)	0.61	2.7	8.5	8.0

- : not reported

To experimentally demonstrate that human erythroids are resistant to the disturbance of heme synthesis and induction of anemia by flumioxazin-induced PPO inhibition, Sumitomo conducted a study with K562 cells, which are derived from human erythroleukemia. They are used as a model for human erythroid maturation since K562 cells can be differentiated into hemoglobin-synthesizing cells by treatment with various inducers. Although accumulation of PPIX resulting from PPO inhibition was observed in K562 cells, no effects were observed on heme content and cell proliferation (Fig. 7) (33) even when treated with 5 μM of flumioxazin. This concentration is close to its water solubility limit (1.79 mg/L) (34). It is also 90 times greater than the concentration of 0.02 ppm (0.056 μM) found in rat embryos from dams treated repeatedly with teratogenic doses of 30 mg/kg (19).

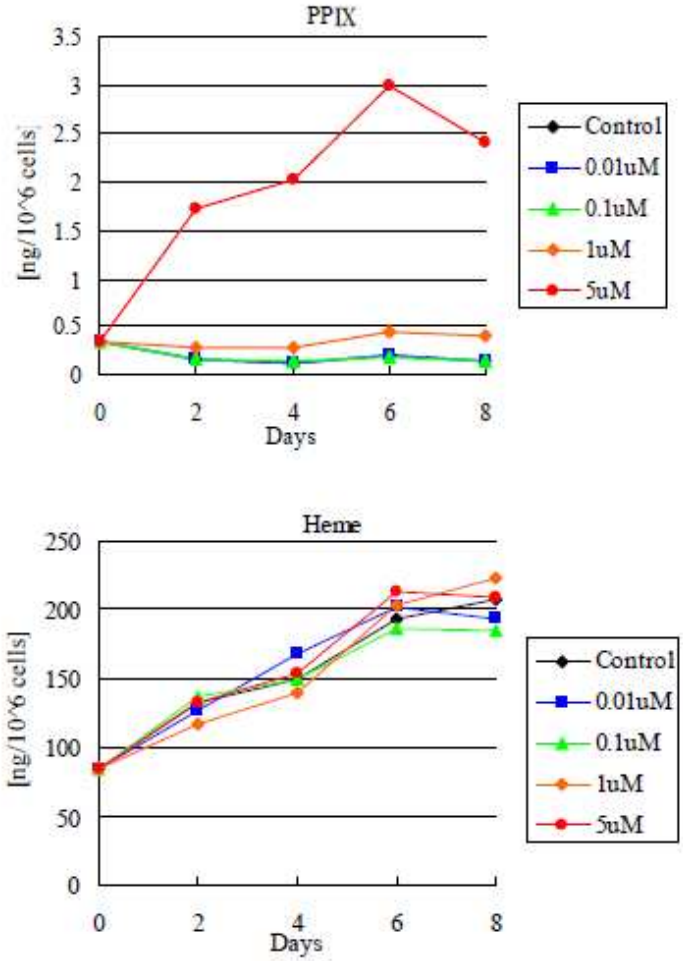

Fig.7 Effects of flumioxazin on PPIX accumulation and heme production of K562 cells

A physiologically based pharmacokinetic (PBPK) model for flumioxazin was developed to predict flumioxazin concentration in the maternal blood and fetus of pregnant human. Flumioxazin concentrations in pregnant rats (30 mg/kg po) were used to develop the PBPK model in pregnant rats using physiological parameters from the literature and chemical-specific parameters from our experimental results and fitting, then, the developed rat PBPK model was extrapolated to the human model. An *in vitro* metabolism study using rat and human liver microsomes was conducted to analyze the species differences in the metabolism of flumioxazin between rat and human. In

addition, a biliary excretion study was conducted in bile duct-cannulated female rats to determine the % absorbance of flumioxazin after oral administration at 1000 mg/kg (35). The developed human pregnant model demonstrated that flumioxazin concentration in the human fetus at dose of 1000 mg/kg po was 0.68 ppm (1.92μM) (36). This concentration is lower than the maximum no effect concentration of 5 μM in K562 cells supporting the view that humans would not be susceptible to anemia and the developmental effects of flumioxazin.

Human erythroblasts are considered to be non-susceptible to flumioxazin when treated at concentrations as high as 5 μM. These concentrations are expected to far exceed those attained in human embryos following flumioxazin exposure.

Moreover, a study with K562 cells was conducted to investigate effects of the three major metabolites including 3OH-flumioxazin, 4OH-flumioxazin, and APF at 5 μM (37). None of the metabolites exhibited any effects on heme and PPIX contents, and cell proliferation although flumioxazin increased PPIX in K562 cells. The results showed that there were no metabolites that could have a more potent effect on human erythroids than flumioxazin.

Clinical and experimental data demonstrate that PPO activity in human erythroids would be higher than the activity of a rate-limiting enzyme in the heme synthetic pathway, and that humans would not become anemic as a result of flumioxazin exposure even in the presence of PPIX accumulation caused by PPO inhibition.

6.3 Synchronous maturation of erythroblasts

In rats, a characteristic of hemopoiesis in yolk sac is that erythroid cells undergo synchronous maturation as a relatively homogeneous population. The morphology and population characteristics of blood cells in rat embryos demonstrated that a vast majority of erythroblasts are polychromatophylic on gestational day 12, the day of the greatest sensitivity, and orthochromatophilic erythroblasts on gestational day 14, when rat embryos were much less sensitive to flumioxazin (Fig. 8) (38).

This explains, in part, why flumioxazin induces an enormous and synchronous loss of blood cells in rat embryos exposed to flumioxazin. In contrast to rats, a relatively heterogeneous population was observed in human primitive hemopoiesis by Kelemen (Fig. 8) (39), who classified the erythroblast into three types. It is conceivable that the type III erythroblast corresponds to the orthochromatophilic erythroblast, and type I and type II correspond to earlier erythroblasts, presumably basophilic or polychromatophilic. Relative populations of type I, II, and III observed in yolk sac range from 7% to 40%, from 21% to 89%, and from 4% to 65%, respectively, during the period

from commencement of human primitive hemopoiesis in week 3-4 to completion of ventricular septum formation in week 8 (40). Thus in humans, even if a particular population is lost, blood cell loss could not be as massive as in rats.

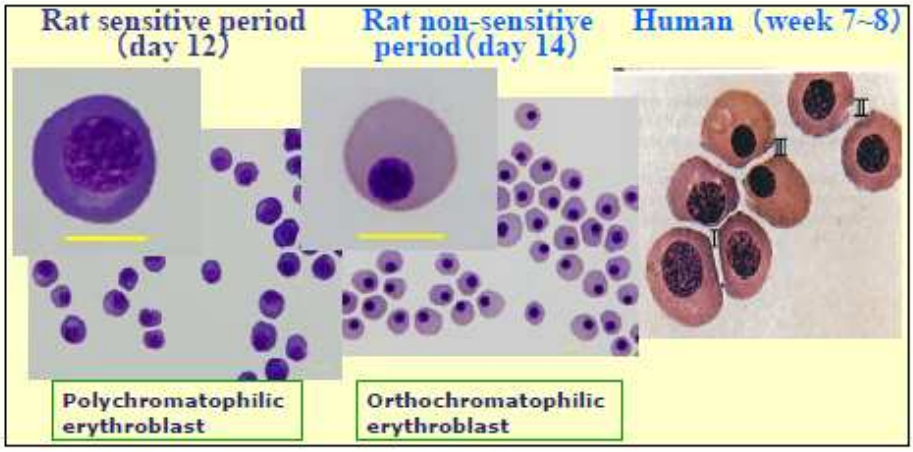

Fig. 8 Erythroblasts in the rat and human embryo

6.4 Vulnerability of erythrocytes exposed to various agents

It is known that rat erythrocytes are more fragile than human erythrocytes when exposed to osmotic imbalance, pH change (41) and oxidative damage (42).

6.5 Relationship between developmental periods of primitive erythropoiesis and interventricular foramen closure

The appearance of blood cell loss and resulting enlarged heart preceding completion of ventricular septum formation is indispensable in VSD induction since VSD does not occur after the completion of ventricular septum formation. Primitive hemopoiesis occurs before and during ventricular septum formation in both rats and humans, demonstrating that there is no species difference at this stage of development.

7. Conclusions

There is convincing evidence for a single mode of action causing the developmental toxicities in rats. The sequence of key biological events in the proposed mode of action has been elucidated.

The studies have lead to the following understanding of the different developmental susceptibilities to flumioxazin in rats and humans, and whether the rat is a relevant model in the assessment of the human hazard of flumioxazin. Overall, it is concluded that the human embryo would be far less sensitive than the rat embryo to the effects of flumioxazin for the following reasons:

7.1 Human PPO is significantly less sensitive to flumioxazin than rat PPO *in vitro* and at the cellular level.

7.2 Decreased PPO activity in rat erythroids results in anemia leading to developmental toxicities. In contrast, reported clinical evidences demonstrate that PPO activity is much higher than a rate-limiting enzyme in the heme biosynthetic pathway in human erythroids. It is therefore very unlikely that the reduction of PPO activity could induce anemia or disturbance of heme synthesis in human erythroids.

7.3 Human erythroblasts are considered to be non-susceptible to flumioxazin when treated at concentrations that are expected to far exceed those attained in human embryos following flumioxazin exposure. Pharmacokinetic modeling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at exposures exceeding a maternal dose of 1000 mg/kg/d.

7.4 Because of a less homogeneous population in primitive erythropoiesis in humans, loss of particular population would not lead to a massive drop in red cells.

7.5 Rat erythrocytes are more fragile than human erythrocytes.

The rat is therefore an inappropriate model for assessing the developmental toxicity of flumioxazin in humans because of species-specific sensitivity to PPO inhibition inducing fetal anemia in rats and consequent developmental toxicity. There is considered to be no plausible scenario whereby humans would be at risk of developmental toxicity given the species difference in susceptibility to flumioxazin and potential for anemia.

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ANNEX 2

AN UPDATE OF A DISCUSSION OF THE MECHANISTIC RESEARCH CONDUCTED ON THE DEVELOPMENTAL TOXICITY OF FLUMIOXAZIN (BASED ON STUDIES PRIOR TO THE PUBLICATION OF FLUMIOXAZIN IN THE 28TH ATP OF THE DSD)

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DATA SUBMISSION VOLUME 45

SBT-0067

SUBMITTED TO SUPPORT THE REGISTRATION OF:

Flumioxazin Technical and V-53482 WP and WDG Herbicides
for Use on Soybeans and Peanuts

DATA REQUIREMENT:

83-3 (OLD)
870.3700 (NEW)

STUDY TITLE:

An Update of a Discussion of the Mechanistic Research Conducted on the Developmental Toxicity of Flumioxazin Technical

AUTHOR:

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and
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STUDY COMPLETED:

May 23, 1997

PERFORMING LABORATORY:

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LABORATORY PROJECT IDENTIFICATION:

482-97-6

STUDY VOLUME:

1 of 1

TOTAL PAGES:

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Statement of No Data Confidentiality Claims

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of **FIFRA** Section 10 (d) (1) (A), (B), or (C).

- This statement supercedes any other claims of confidentially found in this report.

Company: VALENT U.S.A. CORPORATION

Company Agent: Carmella I. Tellone

Title: Manager, Toxicology

Date: May 28, 1997

Signature: Carmella I Tellone

FLAGGING STATEMENT

I have applied the criteria of 40 CFR 158.34 for flagging studies for potential adverse effects to the results of the attached study. This study neither meets nor exceeds any of the applicable criteria.

Company: **VALENT U.S.A. Corporation**

Company Representative: **Carmella I. Tellone, Ph.D., DABT**

Title: **Manager, Toxicology**

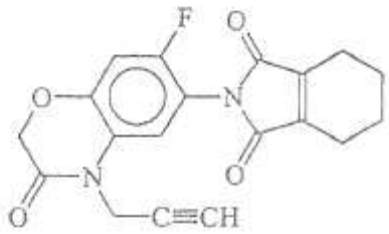
Signature: Carmella I Tellone

Date: May 28, 1997

INTRODUCTION

V-53482 WP Herbicide is under development by Valent U.S.A. for control of broadleaf weeds in soybeans and peanuts. V-53482 WP Herbicide is a low use rate preemergence herbicide which will be packaged as a 51% wettable powder in water soluble bags to minimize exposure to mixer/loaders. The product controls susceptible weeds as a preemergence treatment in conventional, minimum, or no-tillage soybeans and preemergence treatment in peanuts. V-53482 WP Herbicide is applied at 1.5 to 3.0 ounces of formulated product per acre, which is equivalent to 21.7 to 43.4 grams active ingredient per acre. The active ingredient is flumioxazin, 7-fluoro-6-[(3,4,5,6-tetrahydo)phthalimido]-4-(2-propynyl)-1,4-benzoxazin-3(2H)-one.

The chemical structure of flumioxazin is shown below:



Valent U.S.A. previously submitted a series of developmental toxicity studies and a rat reproduction study as part of the supporting documents for both a crop-destruct Experimental Use Permit (EUP) (submission date: February 23, 1993) and a temporary tolerance EUP (submission date: August 3, 1993) with V-53482 WP Herbicide. The submitted studies included an oral rat teratology study (Reference 1), a dermal rat teratology study (Reference 2), an oral rabbit teratology study (Reference 3) and a two-generation rat reproduction study (Reference 4). Detailed summaries of these reports are included in Section C of the Section 3 registration submission.

In essence, it has been shown that Flumioxazin Technical produces developmental toxicity in rats in the absence of maternal toxicity, at doses of 30 mg/kg/day by the oral route and 300 mg/kg/day by the dermal route. The no-observable-effect-level (NOEL) for these studies are 10 and 100 mg/kg/day, respectively. [EPA's selection of NOELs for these studies were 3 and 30 mg/kg/day, respectively, for the oral and dermal rat studies. Valent

provided a rebuttal (MRID No. 43935506) to EPA's choice of NOELs and is awaiting EPA's response.] The developmental effects noted consist primarily of decreased live fetuses and fetal weights, cardiovascular abnormalities (mainly ventricular septal defects [VSD]), wavy ribs and a decreased number of ossified sacrococcygeal vertebral bodies. On the other hand, the response in rabbits was very different from that in rats. Flumioxazin Technical produced no developmental toxicity in rabbits even at a maternally toxic dose of 3000 mg/kg/day. In a very limited study with mice at a dose of 100 mg/kg/day, no developmental toxicity was observed (Reference 5).

The reproductive effect in rats of high doses (200 ppm and greater) of Flumioxazin Technical was observed in the reduction of number of livebom, pup weights, viability index, litter size, and increased clinical and necropsy observations related to pup morbidity for F_1 and F_2 offspring. Reduced epididymal, testes and prostate weights as well as a reduced number of rats mated were noted for F_1 males at 300 ppm, the highest dose tested. Reproductive effects observed for P_1 and F_1 females at 300 ppm related primarily to the loss of litters, pup deaths and an increase in the number of F_1 females that did not deliver a litter. Overall, the lowest NOEL for both generations for systemic and adult reproductive toxicity was 200 ppm and for effects on F_1 and F_2 offspring the NOEL was 100 ppm.

MECHANISTIC STUDIES

After reviewing the results of these studies, Valent U.S.A. and its parent company, Sumitomo Chemical Company, believed the species difference in developmental toxicity was striking and initiated an extensive research program to determine which species was more relevant to risk assessment for humans and develop a better understanding of the mode of action by which flumioxazin produces developmental toxicity in rats. This document is an update of a white paper dated July 21, 1993, discussing the mechanistic research, which was provided to EPA as part of the submission for the temporary tolerance EUP.

An hypothesis was developed, outlined in Figure 1, which postulates the mechanism by which developmental toxicity is produced by flumioxazin. In this scheme, initial research findings indicated that flumioxazin inhibits a key enzyme, protoporphyrinogen oxidase (PPO), in rats, thereby interfering with normal heme synthesis, and resulting in anemia. We postulate that the fetal anemia leads to hypoxia in fetal tissues followed by suppressed liver function and a decrease in protein synthesis. We suggest that the decreased protein synthesis would result in wavy ribs and changes in osmotic forces leads to the edema observed in the fetus. Concurrently, the fetus would compensate for the anemia by pumping a greater volume of blood leading to the observed enlargement of the heart. Thus, we believe the VSD observed in the teratology study is produced by mechanical

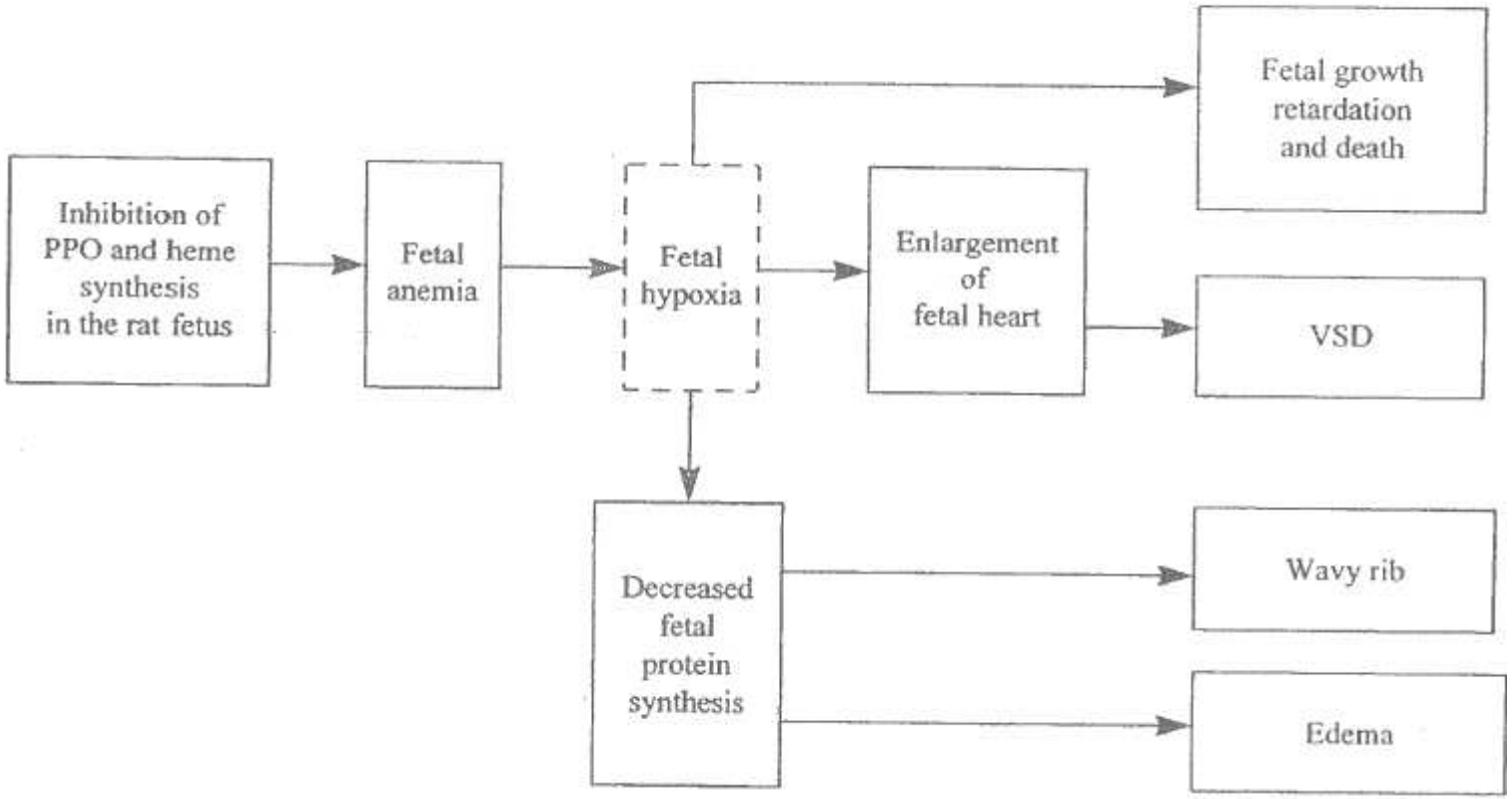


Figure 1: Postulated Mechanism of Developmental Toxicity Produced By Flumioxazin in Rats
(Boxes with solid lines indicate experimental data currently available.)

distortion of the heart. The two other signs of developmental toxicity reported, growth retardation and fetal death, we believe, are also related to the hypoxia produced by the anemic condition in the fetus.

This document will discuss the results from the mechanistic research project which address many parts of the postulated mechanism of production of developmental toxicity in rats following exposure to flumioxazin:

1. Hematotoxicity of flumioxazin in rats
2. Placental transfer of flumioxazin
3. Critical period of embryonic sensitivity
4. Histopathological study of early stages of development in rat and rabbit embryos following exposure to flumioxazin
5. Pathogenesis of developmental effects produced by flumioxazin
6. Protoporphyrin IX (PPIX) accumulation in embryos
   a. Species differences between rat and rabbit embryos
   b. Compound differences in PPIX accumulation in rat embryos
   c. Critical period for PPIX accumulation in rat and rabbit embryos
7. Inhibition of protoporphyrinogen oxidase (PPO) in rat, rabbit and human tissues

The reports of each of these studies are provided as part of the data submission for the Section 3 registration submission for V-53482 WP Herbicide. Studies for which only data summary reports were available at the time of the temporary tolerance submission are now part of Section 3 registration package as full reports.

HEMATOTOXICITY OF FLUMIOXAZIN

Hematotoxicity in the form of reduced hemoglobin level, hematocrit value, mean corpuscular hemoglobin, mean corpuscular volume, myeloid/erythroid ratios, and red blood cell counts, was observed in rats following subchronic dietary exposures of 300 to 3000 ppm of Flumioxazin (Reference 6 and 7). Similar findings of anemia were observed in the chronic/oncogenicity study in rats at doses of 500 and 1000 ppm (Reference 8). The anemia lasted throughout the treatment period, however, it was not progressive nor aplastic in nature. Mean hemoglobin and hematocrit were also reduced in rats in a 21-day

dermal toxicity study at a dose of 1000 mg/kg/day, however, no other signs of hematotoxicity were present (Reference 9).

The rat appeared to be the sensitive species regarding the finding of anemia. No findings indicative of anemia or other type of hematotoxicity were observed in either the four-week subchronic (up to 10000 ppm) (Reference 10) or the 18-month oncogenicity study (up to 7000 ppm) with mice (Reference 11). Anemia was also not observed in dogs during the conduct of the subchronic or chronic studies at doses up to 1000 mg/kg/day (Reference 12 and 13).

A study was designed to elucidate the mechanism by which flumioxazin induces the species-specific anemia in rats (Reference 14). Female Crj:CD (SD) rats were exposed to 3000 or 10000 ppm flumioxazin in the diet for up to 5 weeks. Serial determinations of hematological endpoints, blood biochemistry, urinary coproporphyrin and free erythrocyte protoporphyrin were obtained. Hematological changes at 3000 ppm and the higher dose level included decreased erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and bone marrow myeloid/erythroid ratios. Increases in sideroblasts, erythroblasts and platelets were also noted. Neutrophils and reticulocytes decreased early in the treatment period but increased during the later stages. Urinary coproporphyrin and erythrocyte proporphyrin levels were increased in the 3000 ppm group, the highest dose tested for these endpoints. Treatment-related findings reported for the blood biochemistry analyses include increases in serum iron, total cholesterol, blood urea nitrogen, sodium and potassium, as well as decreases in GOT, uric acid, calcium and triglyceride. Increased liver and spleen weights were also noted.

These findings suggest that the flumioxazin-associated anemia in adult rats can be classified as a sideroblastic anemia. Considering the increases in porphyrins and sideroblasts, the anemia results primarily from interference by flumioxazin in the normal heme pathway during the process of hemoglobin biosynthesis. At the same time, the increase in blood porphyrin level suggests that flumioxazin induces porphyria in rats.

PLACENTAL TRANSFER OF FLUMIOXAZIN

Two placental transfer studies were conducted to determine if the rat fetus was exposed to flumioxazin following an oral dose delivered to the dam, the degree of exposure and the nature of metabolites. In addition the placental transfer studies examined the same parameters in the rabbit and the mouse, two species in which developmental toxicity was not produced by flumioxazin. The purpose of the placental transfer studies was to determine the extent of fetal exposure to flumioxazin as well as whether any species differences existed in this parameter between rats, mice and rabbits.

In the first study, a single oral dose of 30 mg/kg of [phenyl-^{14}C]V-53482 was administered to rats on day 12 of gestation and to mice on day 10 of gestation (Reference 15). ^{14}C-Concentrations in maternal and fetal tissues were measured 1, 2, 4, 8, 24 and 72 hours after administration. ^{14}C-Excretion into urine and feces during 24 hours (mice) or 72 hours (rats) after administration was also examined. Metabolites in excreta, blood cell, plasma, liver and fetus (mouse only) were analyzed in this experiment.

^{14}C-Concentrations in maternal tissues reached maxima and decreased more rapidly in mice than in rats. ^{14}C-Concentration in the fetus reached maxima 1 hour after administration for both species and decreased rapidly thereafter, with half-lives of 14 and 5 hours for rats and mice, respectively. The maximum ^{14}C-concentration in the fetus was 1.05 and 1.72 ppm for rats and mice, respectively.

The excretion of ^{14}C was almost complete within three and one days for rats and mice, respectively. The total ^{14}C-excretion was 95.7% (feces: 74.7% and urine: 21.0%) for rats and 95.8% (feces: 72.9% and urine: 22.9%) for mice.

The metabolism of flumioxazin was qualitatively similar between pregnant rats and mice. However, it appeared that 3-hydroxylation activity was higher in pregnant rats than in pregnant mice. The major metabolite in the mouse fetus at one hour after administration was 4-OH-V-53482.

In the second study (Reference 16), a single oral dose of 30 mg/kg [phenyl-^{14}C]V-53482 was administered to pregnant rats and rabbits on day 12 of gestation. Maternal tissues and fetuses were obtained at 1, 2, 4 and 24 hours after dosing for analysis of ^{14}C concentrations and at 1 or 2 and 24 hours for analysis of metabolites in blood cells, plasma, liver and the fetus.

After 24 hours, the ^{14}C excretion in rabbits was found to be much slower (30.2%) than rats (76.6%). ^{14}C Concentrations in the fetus, amniotic fluid and maternal tissues were greater in the rat than the rabbit with liver and kidney accounting for the greatest concentrations in each species. The maximum ^{14}C-concentrations in maternal tissues were higher than those observed in the fetus or amniotic fluid for both species. Maximum ^{14}C concentrations in the fetus were 0.782 and 0.2 ug equivalents of flumioxazin/g tissue for rat and rabbit, respectively, observed at 4 hours after dosing for both species. For those timepoints where metabolite identification was available for fetal tissue (1 and 24 hours for rats and 2 and 24 hours for rabbits), the highest concentration of parent flumioxazin was 0.06 (at 1 hour after dosing) and 0.02 (at 2 hours after dosing) ug/g tissue for rats and rabbits, respectively.

These data indicate flumioxazin does cross the placenta and that both parent and various metabolites are present in measurable quantities in the fetus. At the same dose level, the concentration of ^{14}C and of flumioxazin is greater in rat fetuses than rabbit fetuses but less than that found in the mouse. No clear pattern of absorption, distribution, metabolism or excretion was evident which could account for the species-specific developmental toxicity in rats.

CRITICAL PERIOD OF EMBRYONIC SENSITIVITY

One of the first steps in our research program on the developmental toxicity of Flumioxazin Technical was to determine the critical period of embryonic sensitivity. To this end, pregnant rats were administered a single dose of 400 mg/kg of Flumioxazin Technical in 0.5% methylcellulose (MC) on one day of gestation beginning on day 11 through day 15 (Reference 17). This dose was selected as one which would produce fetuses with VSD following a single dose without producing excessive fetal deaths. Rats were sacrificed on day 20 and uterine and fetal examinations were performed. Live fetuses were sexed, weighed, examined externally and fixed in Bouin's solution for examination for VSD. Day 12 of gestation was determined to be the most sensitive day based on the incidence of embryonic deaths (39.4%), fetal weight reductions, and incidence of VSD (14%), suggesting a common mechanism for teratogenicity, embryolethality and growth retardation.

In studies of other agents such as x-ray irradiation (Reference 18) and nimustine (Reference 19) which produce VSD, probably by direct damage to the heart via their ability to damage cells, the critical period was determined to be earlier than day 12 of gestation. This suggested to us that flumioxazin might not produce VSD through direct damage to embryonic heart tissue but rather through some indirect means.

HISTOPATHOLOGICAL STUDY OF EARLY STAGES OF DEVELOPMENT IN RAT AND RABBIT FETUSES FOLLOWING EXPOSURE TO FLUMIOXAZIN

As part of the mechanistic research program, an examination of the histopathological changes in rat and rabbit embryos was undertaken (Reference 20). The objective of the study was to look for evidence of direct and/or indirect effects of flumioxazin on embryonic development by a histological examination at light and electron microscopic levels. Pregnant rats and rabbits were administered 1000 mg/kg flumioxazin in 0.5% MC on day 12 of gestation. Control animals received 0.5% MC on day 12 of gestation. Rats were sacrificed at 6, 12, 24, 36 or 48 hours after treatment and rabbits at 6, 24 or 48 hours. Embryos were examined externally and then subjected to examinations of umbilical blood

smears and light or electron microscopic examination of tissues. Umbilical blood smears were stained with Berlin blue for detection of cellular iron. Sagittal or transverse sections of the thoraco-abdominal region of the fetuses were prepared and stained with hematoxylin and eosin for light microscopic examinations. Some sections with heart and liver were stained with Berlin blue and examined. The hearts and livers of some fetuses were prepared for examination by electron microscopy.

No embryonic deaths were observed in rats at 24 hours after treatment. The first intrauterine deaths were observed at 36 hours and embryonic mortality increased to 93.2% at 48 hours after treatment. A primary effect was observed in circulating erythroblasts. Mitochondrial iron deposits in polychromatophilic erythroblasts; dilation of the mitochondrial matrix in polychromatophilic erythroblasts at 6 hours post-dosing; and erythroblastic cell death evident after the appearance of the mitochondrial lesions were observed. These findings suggested the production of anemia in the rat embryo. No histopathological signs in the embryonic rat heart were observed up to 24 hours after treatment, and no cell death in the embryonic rat heart was observed up to 48 hours after treatment suggesting that no primary injury to the embryonic heart was produced by flumioxazin. Histological changes in the rat embryonic hearts at 36 or 48 hours after treatment did include thin ventricular wall; poorly developed ventricular trabeculae; and hypoplasia of the muscular septum and endocardial cushions of the atrioventricular canal.

No treatment-related changes in the external appearance of embryos or intrauterine deaths were produced in rabbits. Likewise, neither iron deposits in erythroblasts of rabbit embryos nor histopathological changes similar to those produced in rat embryos were produced in the rabbit embryo.

Clearly, this study supports the species differences observed in other mechanistic studies and further elucidates the biochemical changes and pathogenesis of the developmental toxicity produced in rats by flumioxazin.

PATHOGENESIS OF DEVELOPMENTAL EFFECTS PRODUCED BY FLUMIOXAZIN

In a continuation of the mechanistic research for flumioxazin, a study was designed to investigate the ontogeny and pathogenesis of a variety of developmental endpoints in rat embryos and fetuses (Reference 21). Pregnant rats received 400 mg/kg of flumioxazin on day 12 of gestation and embryos/fetuses were collected on days 13 through 20 of gestation. Embryos/fetuses were examined externally for enlargement of the heart and edema of the whole body. One half of the litters were examined internally for closure of the interventricular foramen; the other half of the litters were used to measure the number of red blood cells, hemoglobin, and serum protein, and then examined for wavy ribs.

The indicators of developmental toxicity were first apparent on day 14 of gestation when treated embryos were observed to have an enlarged heart, edema and anemia (decreased red blood cell count and hemoglobin). These effects were also observed on days 15 and 16 of gestation, after which the values for treated litters were similar to controls. Beginning on day 15 and continuing to day 20, mortality in the treated litters was increased. The mortality rate was relatively constant throughout this period indicating that all deaths occurred during the earlier period. This is consistent with the early resorptions observed in other developmental toxicity studies with flumioxazin. Closure of interventricular foramen began on day 16 of gestation in control fetuses (72.7% closed). Closure of interventricular foramen did not occur in any treated fetuses on day 16 (0%) and the percentage with closure on day 17 was well below control values (89.7% control vs 23.9% treated). On day 20, the foramen of 95.2% of the control fetuses had closed with only 57.7% of the treated fetuses reaching this milestone. In a separate study, it was determined that the VSD did not resolve during the postnatal period and is a permanent malformation (Reference 22). Serum protein concentration was reduced on days 15 and 16 of gestation in treated litters and recovered by day 17 to values similar to controls. In treated litters, evidence of incomplete chondrification of the ribs was observed on day 16; delayed ossification was observed on day 17; and on day 20 wavy ribs and other skeletal abnormalities were observed.

These data suggest that the enlarged heart, edema, and anemia preceding the occurrence of the fetal mortality may be instrumental in the cause of death. Similarly the occurrence of enlarged heart preceding the failure of the interventricular foramen closure could be related to the pathogenesis of this finding.

PROTOPORPHYRIN IX (PPIX) ACCUMULATION IN EMBRYOS

Flumioxazin is a photobleaching agent whose herbicidal activity is considered to be derived from inhibition of porphyrin biosynthesis, a key step in chlorophyll production in plants (Figure 2). Treatment of plants with flumioxazin results in the accumulation of protoporphyrin IX (PPIX) in plant cells, probably due to inhibition of protoporphyinogen oxidase (PPO) and autooxidation of protoporphyrinogen IX to PPIX (Reference 23). Porphyrin biosynthesis is common to plants and animals as part of chlorophyll and heme synthesis, respectively. Therefore, considering the biological activity of Flumioxazin in plants and indications of hematotoxicity in rats in subchronic (Reference 6 and 7) and chronic toxicity studies (Reference 8), mechanistic studies were conducted examining the accumulation of PPIX in animal embryos exposed to flumioxazin.

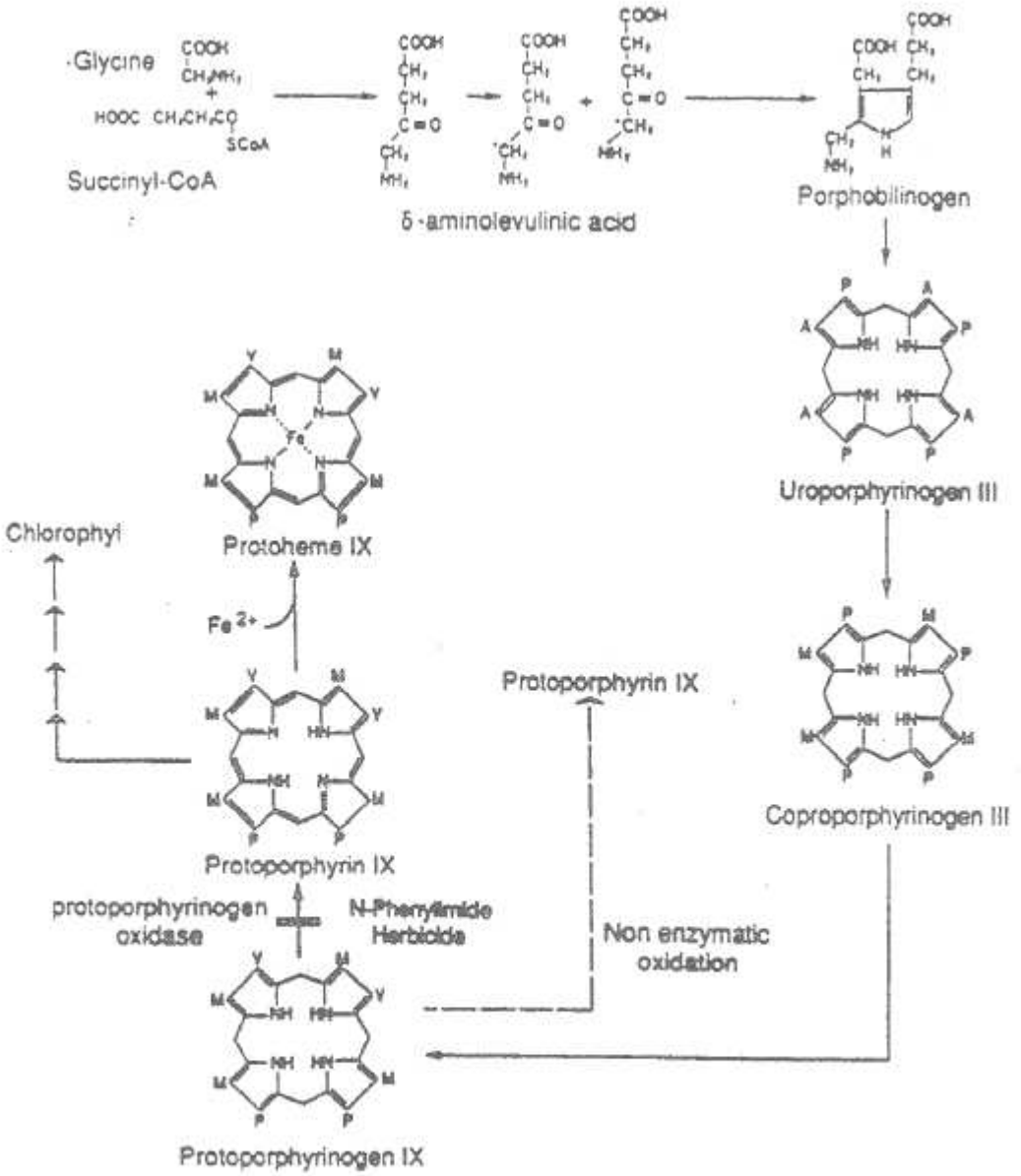


Figure 2. Postulated Mechanism of Action of N-Phenylimide Herbicides

Species Differences Between Rat and Rabbit Embryos

To determine if there was a species difference in accumulation of PPIX in rat and rabbit embryos, pregnant rats and rabbits were administered a single oral dose of 1000 mg/kg flumioxazin in 0.5% MC on day 12 of gestation. The concentration of PPIX in embryos and maternal livers measured at 2, 6, 12, 18 and 24 hours after administration for rats and 2, 6, 12, 24 and 48 hours after administration for rabbits (Reference 24). The concentration in maternal rabbit liver and rabbit embryos was low at all time points. Rats, on the other hand, displayed much higher concentrations with peak PPIX concentrations observed at 12 hours after administration for embryos and at 6-12 hours for maternal livers. Relative to the PPIX concentration in the rabbit tissues, there was approximately a 6-fold greater concentration in maternal rat liver at 12 hours and approximately a 175-fold greater concentration in rat embryos at 12 hours. These data support the hypothesis that there is a biochemical difference in the response of these two species to flumioxazin.

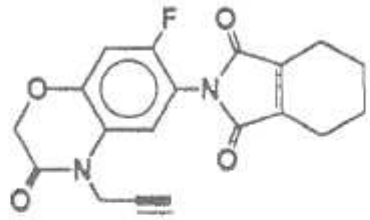
Compound Differences in PPIX Accumulation in Rat Embryos

Three chemically related compounds (flumioxazin, V-23121 and V-23031, see Figure 3) have been tested in standard FIFRA guideline developmental toxicity studies in rat and rabbits. Both flumioxazin and V-23121 were found to produce the same pattern of developmental toxicity in rats (Reference 1 and 25) and were negative when tested in rabbits (Reference 3 and 26). V-23031, however, did not produce developmental toxicity even when tested at 1500 mg/kg/day in rats, a dose well above the test limit dose of 1000 mg/kg/day, or at 800 mg/kg/day in rabbits, a dose which produced maternal toxicity (Reference 27 and 28).

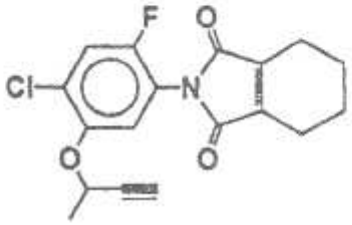
To investigate whether a compound difference in PPIX accumulation was present in rat embryos, pregnant rats were administered 1000 mg/kg of each compound late on day 12 of gestation and PPIX accumulation in whole embryos and maternal livers was measured 14 hours later (Reference 29). The 14 hour time period was selected because it approximates the peak PPIX accumulation time point reported earlier (Reference 24).

Both flumioxazin and V-23121 induced remarkable and similar amounts of PPIX accumulation in rat embryos, with the PPIX concentration in treated embryos more than 250 times that of control fetuses. The PPIX concentration in maternal livers of rats treated with either flumioxazin or V-23121 was approximately three times that observed in livers of control animals. The PPIX concentration in embryos of V-23031 treated rats was similar to the value for control embryos, while the concentration in maternal livers was similar or slightly higher than control. Thus, there is a strong correlation between PPIX accumulation in embryos and the chemicals which were identified as developmental toxicants.

S-53482
(V-53482)



S-23121
(V-23121)



S-23031
(V-23031)

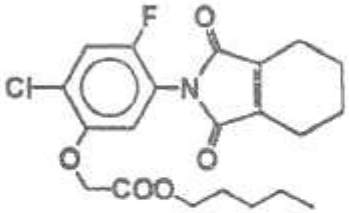


FIGURE 3. Chemical Structures of 3 N-Phenylimide Herbicides.

Critical Period for PPIX Accumulation in Rat and Rabbit Embryos

Further investigations of the correlation of PPIX accumulation in rat embryos in response to exposure to flumioxazin examined whether the critical period for PPIX accumulation was similar to that which was found for the production of developmental toxicity. Pregnant rats received 400 mg/kg flumioxazin and pregnant rabbits received 1000 mg/kg flumioxazin as a single oral dose on one day of gestation beginning on day 10 through day 15 (Reference 30). Animals were sacrificed 14 hours later and the PPIX concentration in embryos was determined. Clear species differences were confirmed, with rat embryos presenting significant increases in PPIX accumulation compared to both control rat embryos and treated rabbit embryos. Peak PPIX concentrations occurred in the treated rat embryos after dosing on days 11 and 12, slightly different from the other critical period study because of the slight difference in time of day at which the dose was administered. Rabbit embryos showed no significant accumulation of PPIX at any time period. The results of this study support the correlation of critical period of developmental toxicity with the critical period for induction of PPIX accumulation in rat embryos exposed to flumioxazin.

INHIBITION OF PROTOPORPHYRINOGEN OXIDASE (PPO) IN RAT AND RABBIT TISSUE

As discussed earlier, PPO is a key enzyme in heme synthesis, catalyzing the transformation of protoporphyrinogen IX to protoporphyrin IX which normally proceeds to formation of protoheme (Figure 2). Three in vitro studies were initiated to determine whether flumioxazin would inhibit PPO in adult liver and/or whole embryos.

In the first study flumioxazin and two chemically related compounds (V-23121 and V-23031, see Figure 3 for structures) were tested for their effect on PPO activity in adult liver, day 12 embryos and day 15 embryos of rats and rabbits (Reference 31). Viable and metabolically active mitochondria were prepared from each of these tissues and incubated with substrate, protoporphyrinogen, and the following concentrations of each test chemical:

Chemical	Concentrations Tested (M)
flumioxazin	10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}
V-23121	10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}
V-23031	10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}

The relative potency for inhibition of PPO from mitochondria of all tissues was flumioxazin> V-23121 >> V-23031. IC_{50} values determined in this study are presented in Table 1. Differences in sensitivity between the two species were observed, with rat tissue exhibiting a greater sensitivity to PPO inhibition by the test chemicals than rabbit tissues. Adult liver and embryo mitochondria showed similar sensitivity to PPO inhibition by the test chemicals, suggesting that adult liver mitochondria could serve as a source of PPO in future

Table 1: Mean IC_{50} Values for Test Chemicals in PPO Assay

Species	Tissue	IC_{50} Values (μM)		
		Flumioxazin	V-23121	V-23031
Rat	Liver	0.00808	0.0108	0.793
Rabbit	Liver	0.0519	1.56	4.75
Rat	Day 12 Embryo	0.0121	0.0467	0.344
Rabbit	Day 12 Embryo	0.0950	6.49	5.92
Rat	Day 15 Embryo	0.00590	0.0200	0.204
Rabbit	Day 15 Embryo	0.308	1.27	5.09

experiments and eliminating the requirement for embryonic tissue. The results of this study further support the hypothesis that the developmental toxicity in rats is related to the interruption of normal heme synthesis by flumioxazin.

Since adult liver was shown to be an adequate surrogate for embryonic tissue in the prior study, only adult liver samples were used in the two studies that followed. The second in vitro study, conducted in the same laboratory as the study cited above, examined effects on PPO activity produced by V-53482 in adult female human liver samples in addition to those from rats and rabbits (Reference 32). This study is unique and probably the most significant study of all the mechanistic studies conducted because for the first time a direct comparison of results with human tissues versus those results with rat tissue could be made.

Mitochondria were prepared from the livers of adult female rats, rabbits and humans. The human liver specimens were acquired through cooperation with organ procurement organizations from brain-dead human organ donors. The inhibition of PPO activity was studied in freshly prepared mitochondria. The final test concentrations were as follows: for human -- 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M; for rat and rabbit -- 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} M. The calculated IC_{50} values are presented in Table 2. The relative sensitivity of the species to PPO inhibition by V-53482 was rat > human > rabbit. The IC_{50} for human liver PPO was 0.0173 ± 0.0044 μM. The IC_{50} determined in the current study for rat liver mitochondria agreed very closely with that determined in the previous study (0.00715 ±0.0021 compared to 0.00808 ± 0.0027 μM reported in Reference No. 33). The IC_{50} for flumioxazin to rabbit liver PPO determined in the present study was slightly higher than the value obtained previously (0.138 ±0.0739 μM compared to 0.0519±0.028 μM). These data are compelling. Given the extent of association of PPO inhibition and the production of developmental effects observed in other studies along with the relative increased sensitivity of the rat compared to human mitochondria to PPO inhibition, Valent concludes that the rat is a conservative surrogate for the human in the risk assessment for flumioxazin.

The third study, conducted in Sumitomo's laboratory under different experimental conditions, examined the effect of flumioxazin and the other two structurally related chemicals on PPO activity in adult female rat and rabbit liver (Reference 31). A summary of the IC_{50} values from all three studies for adult liver samples is presented in Table 2. While there were differences in the absolute values for the IC_{50}s between the two laboratories, the relative effects remained the same. Specifically, rabbit tissue was less sensitive to PPO inhibition than rat tissue and flumioxazin and V-23121, the two agents that produced developmental effects in rats, both produced the greatest inhibition of PPO.

The data from these three studies support the following conclusions:

1. While there was some difference in values between the two laboratories, the relative potency of the three chemicals is flumioxazin > V-23121 >> V-23031.

2. The relative relationship of PPO inhibition between the three species tested is rat > human> rabbit.

Table 2: Mean IC_{50} Values for Test Chemicals in PPO Assays (μM)

Chemical	Rat Liver			Rabbit Liver			Human Liver
	Study #1	Study #2	Study #3	Study #1	Study #2	Study #3	Study #2
Flumioxazin	0.00808	0.00715	0.023	0.0519	0.138	0.30	0.0173
V-23121	0.0108	---	0.036	1.56	---	0.690	---
V-23031	0.897	---	2.23	4.75	---	12.50	---

Study #1: = MRID No. 42884008

Study #2: = Reference 32

Study #3: = Reference 33

3. Using only the IC_{50} values obtained in the same experiment for the three species the relative relationship is still:

rat (0.00715 μM) > human (0.0173 μM) > rabbit (0.138 μM)

Therefore, on the basis of relative sensitivity to PPO inhibition, risk assessments using NOEL for studies in the rat more than adequately protect humans.

SUMMARY AND CONCLUSION

Studies with flumioxazin indicate that this chemical produces developmental toxicity in rats but in not rabbits or mice. An hypothesis is presented in this document which postulates a mechanism for the specific developmental toxicity observed and accounts for the species differences as well. A series of mechanistic studies were conducted to provide support for this hypothesis and demonstrate that the rat is a valid and conservative surrogate for the human in the risk assessment of flumioxazin

We have demonstrated that:

- Flumioxazin interferes with normal heme biosynthesis resulting in sideroblastic anemia and porphyria in adult rats.
- ^{14}C-Flumioxazin administered to pregnant rats on day 12 of gestation crosses the placenta and reaches the rat fetus at maximum levels of ^{14}C and parent, flumioxazin, 4 hours later.
- No clear pattern of absorption, distribution, metabolism or excretion was evident which could account for the species-specific developmental toxicity in rats.
- The critical period for sensitivity to the developmental effects of flumioxazin in rats is day 12 of gestation. This correlates with the peak period of PPIX accumulation in maternal rat liver and the rat fetus.
- A histological examination of rat fetus indicated signs of fetal anemia within 6 hours after dosing but no histological changes in the fetal rat heart were observed until 36 or 48 hours after treatment. No effects were observed in rabbit embryos treated in the same manner as the rats.
- Other observations in the pathogenesis of the developmental effects of flumioxazin in rat fetuses included: enlarged heart, edema, anemia (decreased red blood cell count and hemoglobin), delayed closure of the interventricular foramen, reduced serum protein and incomplete/delayed ossification of the ribs.
- The observation of enlarged heart, edema and anemia preceding the occurrence of fetal mortality suggest these effects may be instrumental in the cause of fetal deaths.

- The occurrence of an enlarged heart preceding the failure of interventricular foramen closure could be related to the pathogenesis rather than a direct toxic effect of flumioxazin on cardiac tissue.

- A strong correlation exists between PPIX accumulation, an indicator of disrupted heme synthesis, and developmental toxicity. Evidence of this correlation exists on the basis of species differences between rats and rabbits; the critical period of sensitivity to developmental effects in the rat; and compound-specific differences with two chemicals structurally related to Flumioxazin, one which produces developmental effects in rats and one which does not.

- Species-and compound-related differences were also observed in the *in vitro* inhibition of PPO, an key enzyme in normal heme synthesis. Based on the relative sensitivity to PPO inhibition in the three species tested (rat>human>rabbit), risk assessments using the NOEL for studies in the rat more than adequately protect humans.

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