Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU 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: 103361-09-7

CLH-O-0000004153-83-03/F

Adopted

06 June 2014

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06 June 2014
CLH-O-0000004153-83-03/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonized classification and labelling (CLH) of:

Chemicals name: 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: 103361-09-7

The proposal was submitted by **Czech Republic** and received by the RAC on **26 August 2013.**

In this opinion, all classifications are given in the form of CLP hazard classes and/or categories, the majority of which are consistent with the Globally Harmonized System (GHS). The classification notation for 67/548/EEC, the Dangerous Substances Directive (DSD) is no longer provided.

PROCESS FOR ADOPTION OF THE OPINION

The **Czech Republic** has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at on 06 September 2013. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **21 October 2013**.

ADOPTION OF THE OPINION OF THE RAC

Rapporteur, appointed by RAC: **Thomasina Barron**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation. The RAC opinion on the proposed harmonized classification and labelling was reached on **06 June 2014** and the comments received are compiled in Annex 2. The RAC Opinion was adopted by consensus.

OPINION OF THE RAC

The RAC adopted the opinion on **Flumioxazin** that should be classified and labelled as follows:

Classification and labelling in accordance with the CLP Regulation

Annex VI	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors
					Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)	
Current Entry	613-166-00-x	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-dicarboxamide	-	103361-09-7	Repr. 1B Aquatic Acute 1 Aquatic Chronic 1	H360D*** H400 H410	GHS08 GHS09 Dgr	H360D*** H410		M=1000
Proposal for RAC	613-166-00-x	flumioxazin (ISO); 2-[7-fluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3(2H)-dione	-	103361-09-7	**Remove:** Repr. 1B	**Remove:** H360D***	**Remove:** GHS08 **Modify:** Wng	**Remove:** H360D***		**Add:** M (chronic) = 1000
RAC opinion	613-166-00-x	flumioxazin (ISO); 2-[7-fluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3(2H)-dione	-	103361-09-7	**Retain:** Repr. 1B	**Retain:** H360D	**Retain:** Dgr	**Retain:** H360D		M (chronic)= 1000
Resulting Annex VI entry if agreed by COM	613-166-00-x	flumioxazin (ISO); 2-[7-fluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3(2H)-dione	-	103361-09-7	Repr. 1B Aquatic Acute 1 Aquatic Chronic 1	H360D H400 H410	GHS08 GHS09 Dgr	H360D H410		 M=1000 M=1000

Annex VI	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors
					Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)	
Current Entry	613-166-00-x	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-dicarboxamide	-	103361-09-7	Repr. 1B Aquatic Acute 1 Aquatic Chronic 1	H360D*** H400 H410	GHS08 GHS09 Dgr	H360D*** H410		M=1000
Proposal for RAC	613-166-00-x	flumioxazin (ISO); 2-[7-fluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3(2H)-dione	-	103361-09-7	**Remove:** Repr. 1B	**Remove:** H360D***	**Remove:** GHS08 **Modify:** Wng	**Remove:** H360D***		**Add:** M (chronic) = 1000
RAC opinion	613-166-00-x	flumioxazin (ISO); 2-[7-fluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3(2H)-dione	-	103361-09-7	**Retain:** Repr. 1B	**Retain:** H360D	**Retain:** Dgr	**Retain:** H360D		M (chronic)= 1000
Resulting Annex VI entry if agreed by COM	613-166-00-x	flumioxazin (ISO); 2-[7-fluoro-3-oxo-4-(prop-2-yn-1-yl)-3,4-dihydro-2H-1,4-benzoxazin-6-yl]-4,5,6,7-tetrahydro-1H-isoindole-1,3(2H)-dione	-	103361-09-7	Repr. 1B Aquatic Acute 1 Aquatic Chronic 1	H360D H400 H410	GHS08 GHS09 Dgr	H360D H410		 M=1000 M=1000

SCIENTIFIC GROUNDS FOR THE OPINION

HUMAN HEALTH HAZARD ASSESSMENT

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.**

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.

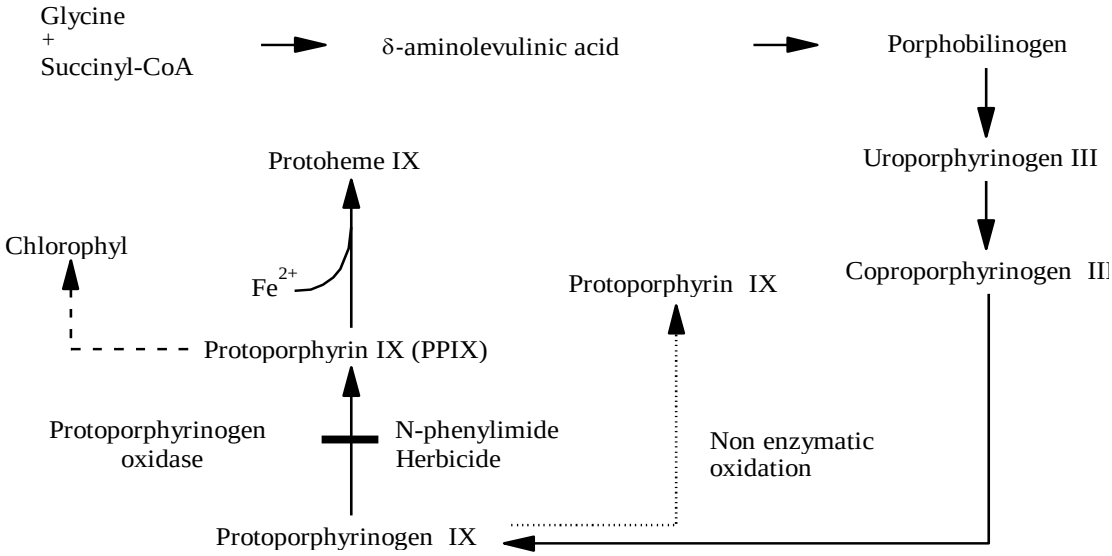
Proposed mechanism of action.

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



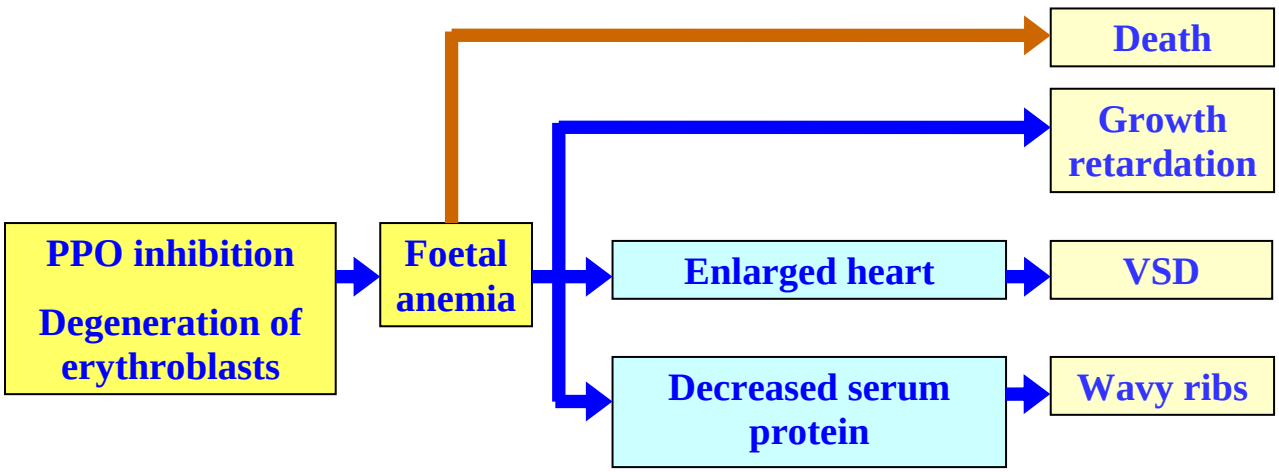


Figure 2. Proposed mechanism of developmental toxicity induced by flumioxazin

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 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**	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 that the S-53482 induces porphyria in rats.	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 **[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 5 females/gp) oral: gavage rat / rabbit: 400 /	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.	Kawamura (1996c) SBT-0063 and Kawamura (1993e)

Method	Results	Reference
1000 mg/kg (exposure: single dose on GD 10 - 15) EPA OPP 83-3;non-GLP **Flumioxazin** **purity: 94.8%**		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.

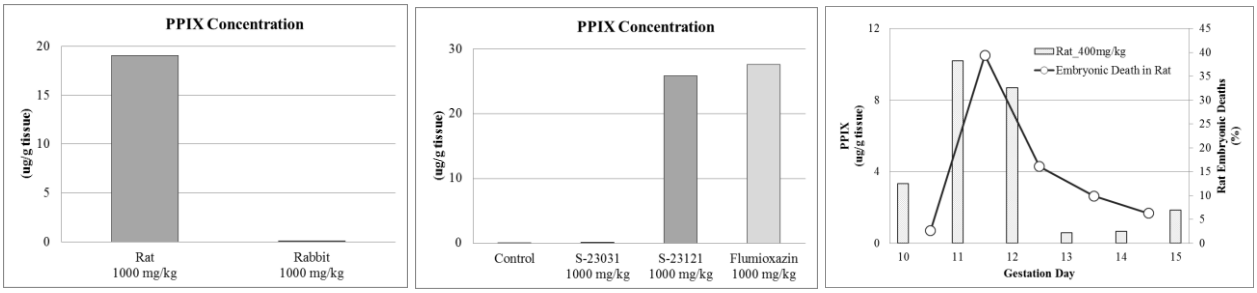


Fig. 3 Close link between PPIX accumulation and developmental toxicity

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, *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.	Abe, J. (2011a) SBT-0118
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	Kawamura, S. (2012a) SBT-0119

	at the highest dose tested.	
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. Species specificity in sensitivity to PPO inhibition

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.

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.

Comparison with the criteria
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

The mechanism is considered plausible on the basis of the following supporting data:
-demonstration of a critical window for all developmental toxicity endpoints
-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.
-histological evidence of mitochondrial lesions in polychromatophilic erythroblasts and cardiac damage in the rat foetus (compared to no effect in rabbits).
-demonstration of the pathogenesis of the developmental effect in rat (no effect in rabbit).
However, there are some remaining uncertainties;
- 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).
- 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.
- 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.

-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.

B. Human relevance:

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

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

ENVIRONMENTAL HAZARD ASSESSMENT

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	*Pseudokirchner-iella 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 $0.0001 < L(E)C_{50} \leq 0.001$ 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**.

Additional references

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Finaurini S, Basilico N, Corbett Y, D'Alessandro S, Parapini S, Olliaro P, Haynes RK, Taramelli D. Dihydroartemisinin inhibits the human erythroid cell differentiation by altering the cell cycle. Toxicology. 2012 Oct 9;300(1-2):57-66.

ANNEXES:

Annex 1 Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in RAC boxes.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and rapporteurs' comments (excl. confidential information).