EUROPEAN CHEMICALS AGENCY

Committee for Risk Assessment

RAC

Annex 2

Response to comments document (RCOM)

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

Triflumizole (ISO); (1*E*)-*N*-[4-chloro-2-(trifluoromethyl)phenyl]-1-(1*H*-imidazol-1-yl)-2-propoxyethanimine

EC number: -
CAS number: 68694-11-1

CLH-O-0000001412-86-40/F

Adopted

04 December 2014

ANNEX 2 - COMMENTS AND RESPONSE TO COMMENTS ON CLH PROPOSAL ON TRIFLUMIZOLE (ISO);(1*E*)-*N*-[4-CHLORO-2-(TRIFLUOROMETHYL)PHENYL]-1-(1*H*-IMIDAZOL-1-YL)-2-PROPOXYETHANIMINE

COMMENTS AND RESPONSE TO COMMENTS ON CLH: PROPOSAL AND JUSTIFICATION

Comments provided during public consultation are made available in this table as submitted by the webform. Please note that some attachments received may have been copied in the table below. The attachments received have been provided in full to the dossier submitter and RAC.

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Substance name: Triflumizole (ISO);(1*E*)-*N*-[4-chloro-2-(trifluoromethyl)phenyl]-1-(1*H*-imidazol-1-yl)-2-propoxyethanimine
CAS number: 68694-11-1
EC number: -
Dossier submitter: the Netherlands

GENERAL COMMENTS

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	Spain		MemberState	1
Comment received				
The Spanish CA agrees with the Dutch proposal for harmonised classification and labelling. Other observations on the CLH Report for triflumizole The Spanish CA noticed the following mistake in the CLH Report for triflumizole: The headline of the acute inhalation toxicity study (p. 27) belongs to the study presented in the DAR of triflumizole (Nishibe et al. 1983d), which was considered not acceptable. This headline should be changed with the information of the study of Janssen, P.J.M. (2005), provided in the Additional Report to the DAR of triflumizole. The reference for this study should also be changed in point 4.2.3 Summary and discussion of acute toxicity (p.28): Janssen, P.J.M., 2005 instead of Nishibe et al. 1983d.				
Dossier Submitter's Response				
Thank you for the correction.				
RAC's response				
The acute inhalation toxicity based on results of the OECD TG 403 acute inhalation toxicity study by Janssen (2005) is assessed by RAC as suggested.				

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	Germany		MemberState	2
Comment received				
The DE CA supports the proposal for harmonized classification and labeling of the NL CA for triflumizole with the exception of the proposed classification as Repr. 1B, H360D. This classification deviates from the previous assessment of EFSA and JMPR. The DE CA considers Repr. 2 more appropriate.				
Dossier Submitter's Response				

Thank you for the support. With regard to the classification for reproduction toxicity, see response to comment 6.
RAC's response
Noted. The classification for reproductive toxicity is based on observations in several studies and include effects like reduced number of live fetuses at birth, increased number of dead fetuses at birth, increased number of late resorptions, reduction on fetal weight and increased placental weight. Based on these findings RAC is of the opinion that classification as Repr. 1B is justified.

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	France		MemberState	3
Comment received				
FR agrees with the classification proposal except for toxicity for the reproduction.				
Dossier Submitter's Response				
Thank you for the support.				
RAC's response				
Noted. The classification for reproductive toxicity is based on observations in several studies and include effects like reduced number of live fetuses at birth, increased number of dead fetuses at birth, increased number of late resorptions, reduction on fetal weight and increased placental weight. Based on these findings RAC is of the opinion that classification as Repr. 1B is justified.				

Date	Country	Organisation	Type of Organisation	Comment number
29.04.2014	Belgium		MemberState	4
Comment received				
We would like to thanks RIVM/SEC for the CLH report on Triflumizole. Editorial comments : Page 27 : We have some doubts about the acute toxicity study via the inhalation route. The explanations are not in accordance with the DAR (part : addendum page 8). The explanation under the short table correspond to another study (Janssen, 2005) presented in the DAR. This study followed the guidance OECD 403. And in the table, it's the Nishibe's study (1983), a study not in accordance with the OECD 403. Page 28 : Same comment, in the table 10, the reference for the inhalation toxicity study is not in accordance with the results.				
Dossier Submitter's Response				
The doubts are correct. Please see our response to comment 1.				
RAC's response				
The acute inhalation toxicity based on results of the OECD TG 403 acute inhalation toxicity study by Janssen (2005) is assessed by RAC as suggested.				

TOXICITY TO REPRODUCTION

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	Spain		MemberState	5

Comment received

p. 73 Summary and discussion of reproductive toxicity

The Spanish CA supports the proposed classification of triflumizole as Repr. 1B, H360D (May damage the unborn child) according to Regulation (EC) 1272/2008; and as T; R61 (May cause harm to the unborn child) according to Directive 67/548/EC.

The main developmental effects observed in the study in rats (Nishibe et al. 1983h), were an increase in the number of late resorptions and increased placental weight, together with a reduction in the number of viable foetuses and in foetal body weight. These effects were seen at the two highest doses. Besides, a skeletal finding (described as 14th rib) appeared to be increased in all dose levels, being statistically significant in the highest dose level.

Triflumizole is an azole compound which presents resemblance in molecular structure and developmental effects with epoxiconazole. Available data on epoxiconazole, shows an increase in late resorptions and enlarged placentae (with placental degeneration) in rats, findings that were related with an aromatase inhibition effect. Epoxiconazole was classified by RAC as Repr. 1B and its classification was supported by letrozole information. Letrozole is an azole compound which is also aromatase inhibitor and induces an increase in late resorptions in rats and an embryotoxic effect in non-human primates.

The available information on these azole compounds tends to demonstrate that late resorptions in rats may be linked to endocrine disruptive effect, including an aromatase inhibiting action in the dams, leading to a depletion of oestradiol. Also, the marked depletion of maternal oestradiol levels can be linked to placental damage and to late foetal death in rats treated with epoxiconazole.

The teratogenicity study with triflumizole in rats does not include any information on hormonal levels to evaluate possible hormonal effects of triflumizole. Data on a possible placental degeneration is not provided either (just data on placental weight is provided).

The similarities on the molecular structure and the developmental effects with the abovementioned azoles make it very likely that the increase in late resorptions with triflumizole were induced via the same mechanism. The lack of information on hormonal levels or placental pathological effects of triflumizole does not allow ruling out the possibility of an endocrine disruptive effect.

On overall, the developmental effects induced by triflumizole, also observed with other azoles with structural analogy, could be due to the same mechanism of action of these azoles: aromatase inhibition. In addition, there is a lack of important information, critical to clarify the mode of action of triflumizole. So, the relevance to humans can not be ruled out. Therefore, as a precautionary approach, the proposal for classification of triflumizole as Repr. 1B (according to Regulation (EC) 1272/2008) and as Toxic for reproduction Cat. 2 (according to Directive 67/548/EC) is supported.

Dossier Submitter's Response

Thank you for the support.

RAC's response

Noted.

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	Germany		MemberState	6
Comment received				
Further toxicological data on Triflumizole was submitted to JMPR (joint meeting on pesticide residues) which was evaluated in 2013 and recently published in a summarized form (http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Report13/JMPR_2013_Report.pdf). Apparently, the toxicological monograph has not been published yet (http://www.who.int/foodsafety/chem/jmpr/publications/monographs/en/). According to JMPR's evaluation, similar effects of post-implantation loss / late resorptions were observed in a second developmental toxicity study in rats (Gotoh, 1986) at 35 mg/kg bw/d (17 vs. 0 for controls, or 4.8 % vs. 0 % for controls) which is not reported in the CLH-dossier for triflumizole. We agree that a classification for developmental toxicity is necessary. Unfortunately, the data presented in the dossier does not provide velar incidences or indications of severities which complicates drawing firm conclusions more difficult. As a teratogenic effect has not been observed and the increased post-implantation loss occurred only at dose levels where maternal toxicity was also seen, we consider classification as Repr. 2 to be more appropriate than Repr. 1B.				
Dossier Submitter's Response				
Thank you for the additional study. In the study by Gotoh, indeed similar effects were observed as in the developmental study by Nishibe (described in the CLH dossier): decrease in the number of live foetuses, decreased fetal weight, increase in placental weight and increase in dead foetuses and resorptions, especially late deaths. This was observed at dose levels of ≥35 mg/kg bw, which in dams also caused a significant reduction in body weight gain (but no significant change in absolute body weight!) and some effects on organ weights. However, changes in organ weights were not definitive of adverse effects of treatment and gross necropsy did not reveal any adverse effects of treatment. It can therefore be concluded that also in this study, it is not likely that the effects on resorptions and fetal death are secondary to maternal toxicity. Also in the study by Nishibe it could be concluded that the effects on maternal body weight were too small to be the cause of an increase in resorptions. A copy of the JMPR (2013) summary of the Gotoh study is added as an Annex to this document. The complete JMPR summmary is available at http://apps.who.int/pesticide-residues-jmpr-database/pesticide?name=TRIFLUMIZOLE The fact that these effects are observed in 2 separate rat studies strengthens the case for classification in Repr. 1B. In addition, teratogenicity is no provision for classification in Repr. 1B. Also effects on development and fetal growth may require classification in Repr. 1B.				
RAC's response				
Since late fetal deaths and reduced viability were observed in several studies RAC is of the opinion that Repr. 1B is justified for developmental toxicity.				

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	Japan	Nisso Soda Co., Ltd	Company-Manufacturer	7
Comment received				

P73-75
ECHA Proposal for Classification of Triflumizole ECHA, as part of a reclassification program for chemicals, are re-examining the reproductive toxicity data for Triflumizole and, on the basis of the late gestational effects in the rat, have proposed a classification of Toxic to Reproduction Category 1B. ECHA give the following reasons for this classification:

1) Similarities in molecular structure and developmental effects (placental enlargement and consequent increased late foetal death) between Triflumizole and Epoxiconazole, an azole fungicide developed by BASF. (The reproductive toxicity data for Epoxiconazole have recently been reviewed by ECHA and Epoxiconazole has been classified as Cat. 1B).

2) Regulatory and investigatory studies with Epoxiconazole, and with Letrozole, an azole compound used to treat breast cancer in humans because of its aromatase inhibitory action, have demonstrated that the placental enlargement and late foetal death in rats are a consequence of oestradiol depletion and that these effects can be ameliorated by co-administration of oestradiol.

3) There is no information to show that the mechanism (endocrine disruption) is not relevant for humans.

Nippon Soda Co., Ltd. (Nisso) disagrees with the proposal for Triflumizole for Reproductive Toxicity Category 1B. However, it is not Nisso's opinion that Triflumizole should be unclassified for reproductive hazard since adverse effects have been clearly demonstrated during the late gestation period in rats. A more appropriate classification for Triflumizole would be Category 2, i.e. as a suspected human reproductive toxicant.
This is based on the report "Expert opinion on the proposed classification of Triflumizole for reproductive toxicity" prepared by Tesh Consultants International (TCI)(2013). A summary of the argumentation which justifies a Category 2 classification for Triflumizole as outlined in the expert opinion report is given below:
Summary
• Epoxiconazole is teratogenic in rats, causing cleft palate and skeletal abnormalities and had already been labelled as Toxic to Reproduction Category 1B because of its teratogenicity. There is no evidence of teratogenicity with Triflumizole in either the rat or the rabbit at dose levels which produce overt maternal toxicity. Triflumizole, therefore, is not considered for classification as a reproductive toxicant on this basis.
• No adverse placental or late gestational effects have been recorded in the rabbit with Triflumizole.
• Masculinisation of female or feminisation of male foetuses/offspring in the rat was not seen following maternal treatment with Triflumizole.
• A clear no-effect level for the late gestational effects in the rat has been established for Triflumizole at x2000 the Theoretical Maximum Daily Intake.
• During more than 25 years of field use of Triflumizole, no questions have been raised regarding the safety upon human reproduction or upon domestic animals or wildlife.
• Tebuconazole, for which the dosing period did not exceed the GD15 threshold demonstrated with Epoxiconazole for inducing late gestational changes, nevertheless showed indications of placental damage and increased post-implantation loss but was not teratogenic. In June 2013, the ECHA Committee for Risk Assessment confirmed Tebuconazole as Hazard Category 2 for Reproductive Toxicity.
• The late gestational effects produced by Epoxiconazole in the rat were shown to be due to oestradiol depletion. However, treatment of pregnant guinea pigs with Epoxiconazole did not give rise to adverse late gestational effects nor to depletion of oestradiol levels.
• The hormonal regulation of pregnancy in the guinea pig is considered to be more similar to that of the human than is the rat, in that the placenta of the guinea pig and the human

assumes the role of oestradiol synthesis in addition to the corpus luteum, whereas the rat relies solely on the corpus luteum for oestradiol synthesis. Thus, the greater capacity of the guinea pig and the human placenta to synthesise oestradiol than the corpus luteum alone may buffer against the effects of aromatase inhibition. The guinea pig, therefore, may be considered a more appropriate animal model than the rat for the extrapolation of effects of aromatase inhibitors on human pregnancy

- The CLH report for triflumizole also made reference to Letrozole, an azole drug used for the treatment of breast cancer in women, where late gestational effects in rats and early pregnancy loss in non-human primates have been demonstrated. However, the relevance of the non-human primate findings in the assessment of Triflumizole reproductive toxicity is questionable since, in the rat, Letrozole is x4000 more potent than Triflumizole in inducing adverse effects in late gestation.
- It is TCI's considered opinion that, for Triflumizole, comparison should be made with Tebuconazole rather than with Epoxiconazole, which is known to be more reprotoxic than either Triflumizole or Tebuconazole, because it is teratogenic in the rat.

Detailed explanations

1) It is important to emphasise one major difference between the developmental toxicity of Triflumizole and Epoxiconazole. Epoxiconazole is a much more potent reproductive toxic agent than is Triflumizole. Triflumizole did not give rise to any teratogenic effects in either the rat or the rabbit, even at dose levels at which maternal performance was adversely affected. Epoxiconazole, conversely, at the top dose level produced a high incidence of cleft palate in the rat, a rare abnormality in this species. The occurrence of abnormalities was independent of changes in oestrogen levels, since co-administration of oestradiol cyclopentylpropionate did not prevent the occurrence of the malformations. Epoxiconazole was, therefore, already classified as Toxic to Reproduction, Category 1B on the basis of teratogenicity. The decision on classification of Epoxiconazole on the basis of late gestational effects in the rat did not modify the previous classification. Triflumizole, however, has not been found to be teratogenic and so classification on the basis of teratogenicity is not an issue. Any classification for Triflumizole will be based solely upon the significance of late gestational effects in the rat.

2) Administration of Triflumizole or Epoxiconazole to the pregnant rat resulted in similar adverse effects in the late gestation period, namely increased foetal death and placental enlargement, at approximately similar dose levels, viz. the lowest adverse effect levels (LOAEL) were found to be 35 mg/kg/day for Triflumizole and 45 mg/kg/day for Epoxiconazole. BASF have performed a series of investigatory studies in the rat with Epoxiconazole in an attempt to explain the aetiology of these findings (Stinchcombe et al. 2013). The combined results of these investigations indicated that administration of Epoxiconazole up to day 15 of gestation (GD15) gave rise to only slight effects in late gestation, whereas when the treatment period was extended beyond GD15 adverse effects, namely late foetal death and placental enlargement, increased in severity in a dose- and time-related manner (Stinchcombe et al. 2013). Taxvig et al (2007, 2008) also investigated the effects of Epoxiconazole in the pregnant rat and found similar effects in late gestation when dosing was continued until GD21. In a detailed study of Epoxiconazole-induced histopathological changes in the rat placenta Moreno et al (2013) demonstrated that the placental enlargement was not due to hypertrophy, but to placental disruption characterised by cystic dilatation of maternal sinuses, and rupture of interhaemal membranes in the labyrinth with concomitant changes in the trophospongium, such as thickening, congestion and necrosis. It was found that there was a direct relationship between the degree of placental damage and foetal survival and this led to the conclusion that the late foetal deaths were a consequence of placental damage rather than to a direct toxic effect of Epoxiconazole upon the foetus. Analysis of maternal hormone levels revealed

marked depletion of oestradiol with less marked changes in progesterone and androstenedione. Co-administration of oestradiol cyclopentylpropionate (ECP) markedly reduced the extent of placental damage and reduced the incidence of late post-implantation loss to that of the controls.
Tiboni et al (2008, 2009) demonstrated similar findings in the rat with Letrozole but at much lower dose levels. They administered Letrozole to pregnant rats between GD6 and GD16 at dose levels between 0.01 and 0.04 mg/kg/day and recorded dose-related increases in both early and late post-implantation losses up to 47% at the high dose level, together with increased placental weight. They found that these effects could be effectively prevented by co-administration of ECP. Comparison of the LOAELs for late gestational effects indicates that Letrozole is approximately x4000 more potent in the rat than either Epoxiconazole or Triflumizole.

3) No endocrine assays were performed in any of the Triflumizole studies but the similarity of the foetal and placental findings with those of Epoxiconazole suggest that a similar mechanism, namely depletion of oestradiol levels, may have been responsible for the adverse changes.
It is pertinent to note that in the rat teratology study with Triflumizole the dosing period conformed to the Japanese regulatory study design in force in the 1980s, i.e. dosing was not terminated on GD15 as for the Western regulatory study design of the time, but continued to GD16. It was reported above for Epoxiconazole that extension of the treatment period into the later stages of gestation exacerbated the effects on the placenta and foetal survival. The Draft Assessment Reports (DAR) for other azole fungicides, for example Bromuconazole, Cyproconazole, Tebuconazole, all contain indications for similar late gestational effects, but differences in study design mean that the evidence is not necessarily so clear-cut. For each of these compounds the treatment period continued only until GD15, in accordance with the Western regulatory study designs. In the light of the Epoxiconazole information, GD15 appears to be on the threshold for inducing late gestational changes. Also, in many studies placental weight was not recorded, and so there is no documented information regarding placental enlargement. Some indirect evidence for placental damage can be seen with Tebuconazole, where, although no placental weights were reported, 9/25 females in the high dose group (120 mg/kg/day) had black/brown fluid in the uterus, associated with increased post-implantation loss (22% compared with 4.6% in the concurrent control group). It is probable that if exposure to Tebuconazole had continued beyond day 15 of gestation then the placental and late gestational effects would have been much more marked. Tebuconazole does not show evidence of teratogenicity and in June 2013, the ECHA Committee for Risk Assessment reconfirmed the classification of Tebuconazole for reproductive toxicity as Category 2 on the basis of the embryofoetal toxicity in the rat. (Ref: CLH-O-0000002717-69-02/F)

4) It was first reported in 1985 that azoles could inhibit the enzyme complex aromatase. Using human placental microsomes with tritiated androstenedione as the substrate, Mason et al. (1985) demonstrated an inhibitory potency ranking of Miconazole > Clotrimazole >> Ketoconazole. Since that time, further work by Mason et al. (1987), Kragie et al (2002)and Trösken et al. (2004) amongst others, using a variety of in vitro assay methods, has extended the list to more than 25 azole compounds, both agrochemicals and pharmaceuticals, all of which have been shown to inhibit aromatase to a greater or lesser degree. Trösken et al. (2004) demonstrated, using an assay based upon human recombinant CYP19 and dibenzylfluorescein as the substrate, that the half maximum inhibitory concentration (IC50) for Letrozole was 0.015, whereas that for Epoxiconazole was 1.44, thus demonstrating a markedly greater potency for Letrozole. Triflumizole was not included in the list of azoles investigated by these workers but because of its chemical class it is likely that it is also an aromatase inhibitor.

Aromatase is the unique enzyme involved in the conversion of androgen precursors to oestrogens. Aromatase is highly conserved amongst mammalian species including humans, and it is the only route by which C19 androgens can be converted to aromatic C18 oestrogenic steroids. Inhibition of aromatase results in an inability to synthesise oestradiol and, if the degree or duration of inhibition is sufficiently severe, it will ultimately result in a depletion of oestrogen levels, which in turn may have adverse effects upon reproductive processes. Theoretically, therefore, because azoles as a class are aromatase inhibitors, all have the potential to reduce oestrogen synthesis and thus all have the potential to present a reproductive hazard. However, in an in vivo situation, there are many factors that can influence the extent to which the aromatase inhibitory effects are manifest. As mentioned above, the level and duration of the exposure is critical but no less important is the reproductive physiology of the species that is exposed to the aromatase inhibitor.

5) BASF considered the influence of species variation when they carried out their investigatory studies with Epoxiconazole. In addition to the rat and the rabbit, they carried out reproductive toxicity studies in the guinea pig, chosen because the reproductive endocrinology of the guinea pig is considered to be more similar to the human situation that that of rodents (see later). Schneider et al (2013) evaluated the effects of Epoxiconazole when administered to guinea pigs during pregnancy. In a pre-natal toxicity study, dosing commenced on GD 6 and was continued throughout organogenesis and the late gestation period until termination on GD 63, whilst in a pre- and post-natal toxicity study dosing commenced on GD 6 and continued until post-natal day 21. In the pre-natal toxicity study, dose levels up to 90 mg/kg body weight/day had no adverse effect upon post-implantation survival, there were no late foetal deaths and placental weight and placental histology were unaffected by treatment. A low incidence of fetuses with minor skeletal changes was recorded but there was no evidence of craniofacial malformation. In the pre- and post-natal toxicity study there were no adverse effects on parturition or post-natal survival and 50 development of offspring. Maternal hormone levels were assayed and although some variation in oestradiol levels was recorded between the two studies, oestradiol levels within each study appeared unaffected by maternal treatment with Epoxiconazole. Changes in circulating levels of adrenal hormones and in adrenal histology were observed, particularly in the pre-natal toxicity study, but these had no impact upon reproductive parameters. Triflumizole, to-date, has not been evaluated in guinea pigs. It should not be overlooked, however, that when Triflumizole was administered to pregnant rabbits during organogenesis there was no evidence of increased post-implantation loss and placental weights were slightly decreased, not increased as in the case of the rat. On the basis of these findings it is clear that there are marked species differences in the response of pregnant animals to the administration of high doses of an aromatase inhibitor. The question has to be asked, therefore, which species is most relevant to the human situation?

In terms of hormonal regulation of pregnancy, the guinea pig is considered to be a more relevant model for the human situation than is the rat, for the following reasons:
In the rat, the corpus luteum, which is rich in aromatase (Keyes et al. 1979), is the only source of oestradiol during pregnancy. The placenta does not express aromatase (Nakanishi 2007). Oestradiol is synthesised in the corpus luteum from androgens which are provided by the placenta. Circulating levels of oestradiol act via a feedback mechanism to regulate the production of androgens, particularly androstenedione (Jackson and Albrecht 1986). Oestradiol levels are relatively low during the early gestation period but increase markedly during the latter stages of gestation (Keyes et al. 1979). The conversion of androstenedione to oestradiol is catalysed by aromatase; thus inhibition of aromatase will reduce or prevent the synthesis of oestradiol. It should be noted that in the rat, treatment with Epoxiconazole was without effect in the early gestation period when oestradiol levels are naturally low, but effects were seen in late pregnancy when the expected surge in

oestradiol levels did not occur.

In the guinea pig and the human for approximately the first 14 days of pregnancy the ovary produces oestradiol and progesterone, but after this time the extra-ovarian sites such as the uterine tissue and placenta begin to synthesis oestrogens and progesterone. Although in normal pregnancies in the guinea pig the ovaries continue to be the major source of these hormones (Batra et al. 1980) the ovaries can be excised in both the human and the guinea pig after this time without altering the course of the pregnancy (Schofield 1960). Kaufmann (2004), in his treatise on the guinea pig placenta, comments "Similar to the human, the endocrine activity of the corpus luteum is taken over by the placenta during the course of pregnancy. Lutectomy in the middle and in the second half of guinea pig gestation are not followed by abortion." Thus it would appear that extra-ovarian sites can synthesize adequate amounts of these hormones to maintain pregnancy. In the human, approximately two months into pregnancy the placenta takes over steroid production (Stocco 2012). Mitchell and Taggart (2009) state that there is similarity between the guinea pig and human in that there is significant steroid production and metabolism not only in the placenta but also within the foetal membranes. In humans, the foetal-placental unit becomes the primary source of oestrogen production, making ovarian steroidogenesis unnecessary (Stocco 2012) Compared with size of the corpus luteum the placenta is very much larger and it could be argued, therefore, that the placenta has a much greater capacity for oestrogen production and can, therefore, provide a better buffer against the effects of aromatase inhibition. In addition in both the guinea pig and the human the chorion and decidua contain sulfohydrolase activity, which catalyses the conversion of oestrone to oestradiol, and they synthesize increasing amounts of oestradiol via this pathway around the time of parturition. (Glutek and Hobkirk 1990), Chibbar et al. 1986). It is worth noting at this point that Stocco (2012) reports that "despite very low levels of oestrogen, pregnancy is not interrupted in all animals treated with aromatase inhibitors, suggesting that placental aromatase is not essential for the development of the foetus. This conclusion is supported by successful pregnancies in women with unusually low oestrogen levels as a result of sulphatase or aromatase deficiencies". Stocco cites France (1979) and Belgorosky et al (2009) in support of this.

Following the mechanistic studies performed in rats and guinea pigs, BASF concluded that the rat findings were species specific and that the guinea pig is the more appropriate model. Annex 5 to the RAC Opinion of toxicity to reproduction of Epoxiconazole (adopted 28 November 2012) includes an expert opinion on the Classification of Epoxiconazole for Developmental Toxicity prepared by Exponent International, Harrogate, UK. One of the authors, Dr. John De Sesso, is a world renowned expert on placentation, and Exponent confirms the view that the guinea pig is a model more representative of human pregnancy than the rat and agrees with the position that these findings are likely species-specific to small rodents and that the new data clearly represent "mechanistic data that raises doubt about the relevance of the effects to humans".

6) Amongst the rapporteurs to ECHA for Epoxiconazole the Swedish CA concluded that the new data in guinea pigs demonstrate dose-dependent effects on adrenal hormones, weights and pathology and thus demonstrate some endocrine disrupting effects. However, they stated that, in view of the known differences in hormonal regulation of late pregnancy between rats on the one hand and guinea pigs and humans on the other, the demonstrated 'placental mechanism' of action for the induction of late foetal resorptions in rats may question the relevance to humans. In the "Comparison with Summary Section 4 - Criteria for Classification" they agree that the mechanistic findings behind the post-implantation loss and resorptions in rats are reason to doubt human relevance of these findings and that these effects do not warrant classification.

Other rapporteurs expressed different views, and took into consideration the work of Taxvig et al (2007, 2008) who demonstrated that maternal treatment with Epoxiconazole not only caused the late gestational effects but also gave rise to virilisation of female pups, and feminisation of male pups. It should be noted, however, that in the Triflumizole rat multigeneration study and in the rat teratology study, even at the highest dose level of 120 mg/kg/day where effects on the placenta and late foetal survival were clearly apparent, there was no indication of modification of external or internal genitalia of foetuses or offspring exposed to Triflumizole in utero.

7) There do not appear to be any reports of azole fungicides that are used in agriculture having been tested in pregnancy in non-human primates, although the effects of Letrozole have been investigated in the baboon. Albrecht et al. (2000) administered 2 mg/animal of Letrozole by the subcutaneous route to pregnant baboons beginning on either GD 30, GD 60 or GD 100 until the animals miscarried or until the foetus was delivered by Caesarian section on GD 160 – 168. Additional animals were coadministered oetradiol (2 mg/animal) during the same treatment periods. Oestradiol levels were monitored throughout the study. They observed that Letrozole administration alone reduced maternal serum oestradiol levels from the untreated mean value of 0.35 ng/ml to a mean value of 0.96 ng/nl, with a consequent loss of approximately 50 % of pregnancies. Co-administration of oestradiol prevented the pregnancy losses. The same group of workers, however, subsequently reported that an approximately similar dose of Letrozole, administered from GD 100 to term, which produced a similar degree of oestradiol depletion, was not associated with any late pregnancy loss unlike the 25% late loss that was seen in the previous study (Aberdeen et al. 2010). However, the relevance of the primate findings with Letrozole to considerations of Triflumizole reproductive toxicity is questionable, since Letrozole was found to be approximately x4000 more potent than Triflumizole in inducing adverse effects in pregnancy in the rat.

8) In terms of other manifestations of endocrine disruption upon reproductive parameters, administration of Triflumizole before mating and during the pre- and post-natal developmental period throughout three generations did not demonstrate adverse effects upon mating performance or fertility of parent animals at dose levels between 8 and 16 mg/kg/day depending upon the age of the animals.
There is, therefore, conflicting evidence and there are conflicting opinions concerning the relevance of the findings in experimental animals to human pregnancy. Endocrine disruption, per se, is not sufficient reason to classify a compound as a reproductive toxicant. There has to be evidence of adverse effects on reproductive processes. It has been shown that considerable species variation occurs in response to administration of azole aromatase inhibitors and there is room to debate the relevance to humans of the positive and negative findings in the different species.

9) In the case of Triflumizole, the adverse findings in rats occurred at extremely high multiples of calculated human exposure levels, and more than 25 years of field use of Triflumizole have not raised any questions regarding the safety upon human reproduction from agricultural workers using Triflumizole or from the general population consuming crops that have been sprayed with Triflumizole.

In conclusion, taken into consideration the above argumentation a more appropriate classification for Triflumizole would be Category 2, i.e. as a suspected human reproductive toxicant, rather than category 1B, i.e. a presumed reproductive toxicant, as currently proposed by ECHA.

References

Aberdeen GW, Baschat AA, Harman CR, Weiner CP, Langenberg PW, Pepe GJ, Albrecht ED (2010): Uterine and fetal blood flow indexes and fetal growth assessment after chronic estrogen suppression in the second half of baboon pregnancy. Am. J. Physiol. 298(3) pp. H881-H889 (http://ajpheart.physiology.org/content/298/3/H881.long)

Albrecht ED, Aberdeen GW, Pepe GJ (2000): The role of estrogen in the maintenance of primate pregnancy. Am. J. Obstet. Gynecol. 182(2) pp. 432-438

Batra S, Sjöberg N-O, Thorbert G (1980): Sex steroids in plasma and reproductive tissues of the female guinea pig. Biol. Reprod. 22 pp. 430-437 (http://www.biolreprod.org/content/22/3/430.long)

Belgorosky A. Guercio G, Pepe C, Saraco N, Rivarola MA (2009): genetic and clinical spectrum of aromatase deficiency in infancy, childhood and adolescence. Horm. Res. 72 pp. 321-330 (cited by Stocco (2012), see below) (http://www.karger.com/Article/FullText/249159)

Chibbar R, Hobkirk R, Mitchell BF (1986): Sulfohydrolase activity for estrone sulfate and dehydroepiandrosterone sulfate in human fetal membranes and deciduas around the time of parturition. J. Clin. Endocrin. Metab. 62(1) pp. 90-94

France JT (1979): Steroid sulphatase deficiency. J. Steroid Biochem. 11 pp. 647- 651 (cited by Stocco (2012), see below)

Glutek SM, Hobkirk R (1990): Estrogen sulfatase and steroid sulfatase activities in intrauterine tissues of the pregnant guinea pig. J. Steroid Biochem. Mol. Biol. 37(5) pp. 707-715

Jackson JA, Albrecht ED (1986): Estrogen regulates placental androstenedione production during rat pregnancy. Endocrinology 119(3) pp. 1052-1057

Kaufmann P (2004): The guinea pig placenta (http://placentation.ucsd.edu/guinea.htm)

Keyes PL, Yuh KC, Miller JB (1979): Estrogen action in the corpus luteum. Adv. Exp. Med. Biol. 112 pp. 447 – 463

Kragie L, Turner SD, Patten CJ, Crespi CL, Stresser DM (2002): Assessing pregnancy risks of azole fungicides using a high throughput aromatase inhibition assay. Endocr. Res. 28(3) pp. 129-140

Mason JI, Murry BA, Olcott M, Sheets JJ (1985): Imidazole antimycotics: inhibitors of steroid aromatase. Biochem. Pharmacol. 34 pp. 1087-1092

Mason JI, Carr BR, Murry BA (1987): Imidazole antimycotics: selective inhibitors of steroid aromatisation and progesterone hydroxylation. Steroids 50(1-3) pp. 179-189

Mitchell BF, Taggart MJ (2009): Are animal models relevant to key aspects of human parturition? Am. J. Physiol. - Regu. Physiol. 297(3) p. R525-R545 (http://ajpregu.physiology.org/content/297/3/R525.long)

Moreno MCR, Fussell KC, Gröters S, Schneider S, Strauss V, Stinchcombe S,Feggert I, Veras

M, van Ravenzwaay B (2013): Epoxiconazole –induced degeneration in rat placenta and the effects of estradiol supplementation. Birth Defects Research (Part B) 98(3) pp. 208-221

Nakanishi T (2007): The problem of species comparison of developmental toxicity: can we extrapolate human developmental toxicity induced by environmental chemicals from data on rodents? Yakugaku Zasshi 127(3) pp.491-500 (read in abstract only – full text in Japanese) (https://www.jstage.jst.go.jp/article/yakushi/127/3/127_3_491/_pdf)

Schneider S, Hofmann T, Stinchcombe S, Moreno MCR, Fegert I, Gröters S, Fabian E, Thiaener J, Fussel KC, van Ravenzwaay B (2013): Species differences in developmental toxicity of Epoxiconazole and its relevance to humans. Birth Defects Research (Part B) 98(3) pp. 230-246

Schofield BM (1960): Hormonal control of pregnancy by the ovary and placenta in the rabbit. J. Physiol. 151 pp. 578-590 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1363285/pdf/jphysiol01286-0178.pdf)

Stinchcombe S, Schneider S, Fegert I, Moreno MCR, Strauss V, Gröters S, Fabian E, Fussel KC, Pigott GH, van Ravenzwaay B (2013): Effects of estrogen coadministration on Epoxiconazole toxicity in rats. Birth Defects Research (Part B) 98(3) pp. 247-259

Stocco C (2012): Tissue physiology and pathology of aromatase. Steroids 77(1-2) pp. 27 – 35 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3286233/)

Taxvig C, Hass U, Axelstad M, Dalgaard M, Boberg J, Andeasen HR, Vingaard AM (2007): Endocrine disrupting activities in vivo of the fungicides Tebuconazole and Epoxiconazole. Toxicological Sciences 100(2) pp, 464-473 (http://toxsci.oxfordjournals.org/content/100/2/464.long)

Taxvig C, Vingard AM, Hass U, Axelstad M, Metzdorff S, Nelleman C (2008): Endocrine disrupting properties in vivo of widely used azole fungicides.Int. J. Androl. 31(2) pp. 170-177

Tesh JM, Tesh SA (2013): Expert Opinion on the Proposed Classification of Triflumizole for Reproductive Toxicity Nisso Report No. RD-02785

Tiboni GM, Marotta F, Rossi C, Giampietro F (2008): Effects of the aromatase inhibitor letrozole on inutero development in rats. Human Reproduction 23(8) pp. 1719-1723 (http://humrep.oxfordjournals.org/content/23/8/1719.long)

Tiboni GM, Marotta F,Castigliego AP, Rossi C (2009): Impact of estrogen replacement on letrozole-induced embryopathic effects. Human Reproduction 24(11) pp.2688-2692 (http://humrep.oxfordjournals.org/content/24/11/2688.long)

Trösken ER, Scholz K, Lutz RW, Völkel W, Zarn JA, Lutz WK (2004): Comparative assessment of the inhibition of recombinant human CYP19 (aromatase) by azoles used in agriculture and as drugs for humans. Endocr. Res. 30(3) pp.387-394

(ECHA note: The following attachments were provided with the comment above)

1. Albrecht ED, Aberdeen GW, Pepe GJ (2000): The role of estrogen in the maintenance of primate pregnancy. Am. J. Obstet. Gynecol. 182(2) pp. 432-438

2. Chibbar R, Hobkirk R, Mitchell BF (1986): Sulfohydrolase activity for estrone sulfate and dehydroepiandrosterone sulfate in human fetal membranes and deciduas around the time of parturition. J. Clin. Endocrin. Metab. 62(1) pp. 90-94

3. France JT (1979): Steroid sulphatase deficiency. J. Steroid Biochem. 11 pp. 647- 651

4. Glutek SM, Hobkirk R (1990): Estrogen sulfatase and steroid sulfatase activities in intrauterine tissues of the pregnant guinea pig. J. Steroid Biochem. Mol. Biol. 37(5) pp. 707-715

5. Jackson JA, Albrecht ED (1986): Estrogen regulates placental androstenedione production during rat pregnancy. Endocrinology 119(3) pp. 1052-1057

6. Keyes PL, Yuh KC, Miller JB (1979): Estrogen action in the corpus luteum. Adv. Exp. Med. Biol. 112 pp. 447 – 463

7. Kragie L, Turner SD, Patten CJ, Crespi CL, Stresser DM (2002): Assessing pregnancy risks of azole fungicides using a high throughput aromatase inhibition assay. Endocr. Res. 28(3) pp. 129-140

8. Mason JI, Murry BA, Olcott M, Sheets JJ (1985): Imidazole antimycotics: inhibitors of steroid aromatase. Biochem. Pharmacol. 34 pp. 1087-1092

9. Mason JI, Carr BR, Murry BA (1987): Imidazole antimycotics: selective inhibitors of steroid aromatisation and progesterone hydroxylation. Steroids 50(1-3) pp. 179-189

10. Moreno MCR, Fussell KC, Gröters S, Schneider S, Strauss V, Stinchcombe S,Feggert I, Veras M, van Ravenzwaay B (2013): Epoxiconazole –induced degeneration in rat placenta and the effects of estradiol supplementation. Birth Defects Research (Part B) 98(3) pp. 208-221

11. Schneider S, Hofmann T, Stinchcombe S, Moreno MCR, Fegert I, Gröters S, Fabian E, Thiaener J, Fussel KC, van Ravenzwaay B (2013): Species differences in developmental toxicity of Epoxiconazole and its relevance to humans. Birth Defects Research (Part B) 98(3) pp. 230-246

12. Stinchcombe S, Schneider S, Fegert I, Moreno MCR, Strauss V, Gröters S, Fabian E, Fussel KC, Pigott GH, van Ravenzwaay B (2013): Effects of estrogen coadministration on Epoxiconazole toxicity in rats. Birth Defects Research (Part B) 98(3) pp. 247-259

13. Taxvig C, Vingard AM, Hass U, Axelstad M, Metzdorff S, Nelleman C (2008): Endocrine disrupting properties in vivo of widely used azole fungicides.Int. J. Androl. 31(2) pp. 170-177

14. Trösken ER, Scholz K, Lutz RW, Völkel W, Zarn JA, Lutz WK (2004): Comparative assessment of the inhibition of recombinant human CYP19 (aromatase) by azoles used in agriculture and as drugs for humans. Endocr. Res. 30(3) pp.387-394

Dossier Submitter's Response

It should be noted that the main reason for classification as Repr. 1B are the effects that are observed in a developmental rat study (decreased no of live fetuses, increased

incidence of late resorptions, together with placental damage). As indicated in comment 6, similar effects were also observed in a second developmental study in rats. I.e., there is clear evidence for developmental effects from two independent animal studies and in principle (without information that the effects are not relevant for humans), this requires classification as Repr 1B. The data on epoxiconazole and letrozole, i.e. on MoA, are only used as supportive evidence, providing a possible explanation for the mechanism. Nevertheless, we agree that this could be emphasized more clearly in the conclusion.

Below, the comments raised by Nisso Soda Co., Ltd will be discussed.

1. We agree that in contrast to epoxiconazole, triflumizole does not induce teratogenic effects. However, this is no provision for classification as Repr 1B, which may also be required due to significant late gestational effects as observed for triflumizole. For expoxiconazole, RAC concluded in 2010 that induction of post-implantation loss is in agreement with the criteria for CLP classification Repr. Cat. 1B.
2. Indeed, for epoxiconazole, mechanistic data are available, indicating aromatase inhibition to be the cause of the late gestational effects. For triflumizole, such studies are not available. In addition, the cause of increased placental weight is unknown. Although we agree that another mechanism cannot be excluded, it is striking that the 2 substances (or even 3 when letrozole is also taken into account) that are structurally related, show similar late gestational effects.
3. In several of the studies reported for tebuconazole (rats,rabbits and mice), placental weight was measured, however, no changes were observed. This indicates that the mechanisms for developmental effects of tebuconazole and epoxiconazole/triflumizole differ or that the differences are caused by the differences in exposure period in the rat study. Furthermore, the classification for tebuconazole for reproductive toxicity was not confirmed in June 2013. It was not discussed as can be seen in the RAC conclusion since no proposal to change the current harmonised classification for this hazard class was proposed. (RAC only advices on the proposed changes but not on the other hazard classes).
4. We agree that level and duration of exposure may influence the effects of aromatase inhibition. However, classification is based on hazards, and exposure levels should not be taken into account.
5. Clearly there are species differences for the effects of triflumizole and epoxiconazole. However, for epoxiconazole it was already concluded that, despite the additional data from the expert opinion on the classification for epoxiconazole, there are not enough data that show the mechanism (endocrine disruption) observed in rats (but not guinea pigs) is not relevant for humans and epoxiconazole was classified as Repr 1B.
6. We agree that for triflumizole, endocrine disruption is only a possible mechanism for the developmental effects, but not a proven mechanism. This strengthens the case that relevance for humans cannot be excluded.
7. Despite the difference in potency, these primate studies show that pregnancy loss also occurs in primates when animals were exposed at the right time, and that it can be prevented by co-administration of oestradiol. Also this point strengthens the case that relevance for humans cannot be excluded.
8. The dose levels used in the 2 generation study (max 16 mg/kg bw) is lower than the doses that induced developmental effects (35 mg/kg bw). This is not conflicting. Although we agree that endocrine disruption on its own may not be enough for classification for reproduction toxicity, two separate rat studies have shown clear effects on late resorptions, placental damage, no of live pups and fetal weight. Since there is no adequate information that these effects are not relevant for humans (despite the fact that they were not observed in another species) there is enough reason for classification as Repr. 1B.

As mentioned before, classification should be based on hazards, not on risks, i.e. exposure levels.
RAC's response
Noted. The classification for reproductive toxicity is based on observations in several studies and include effects like reduced number of live fetuses at birth, increased number of dead fetuses at birth, increased number of late resorptions, reduction on fetal weight and increased placental weight. Based on these findings RAC is of the opinion that classification as Repr. 1B is justified.

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	France		MemberState	8
Comment received				
p.73-74: Based on the effects observed in the 2-generation study (e.g. increased gestation length in the two generations, decreased fertility and conception rate) and the placental effects leading to foetal mortality in the rat teratogenicity study, a classification as Repr. 2 H361f according to CLP or Repr Cat 3 R62 according to DSD could be appropriate. As for epoxiconazole, late resorptions and post-implantations losses were observed with triflumizole. For epoxiconazole, these effects may be linked to endocrine disruptive effects including an aromatase inhibition in the dams leading to a depletion of oestradiol and it was finally agreed that the relevance to humans could not be ruled out. Moreover, skeletal variations and malformations, particularly cleft palates, were observed in the rats. Concerning triflumizole, no mechanistic data are available (e.g. hormonal levels). It is considered that comparison of triflumizole with epoxiconazole and letrozole may not be relevant, as triflumizole is an imidazole whereas the two other are triazoles. Moreover, no skeletal malformations were observed. It is thus considered that a classification for developmental effects as Repr. 2, H361d (CLP) or Repr Cat. 3 R63 (DSD) could be sufficient.				
Dossier Submitter's Response				
The decreased fertility and conception rate in the F1 (first mating) was not statistically significant and not repeated in the second mating or in the parental animals. Therefore, this is considered not sufficient for classification for fertility. The placental effects and the foetal mortality are considered developmental effects. Similar effects on late resorptions, placental weight, viable foetuses and fetal body weight are observed in two rat studies (see also comment 6), both at dose levels with only minimal maternal toxicity. It can thus be concluded that there is 'clear evidence of an adverse effect on development in the absence of other toxic effects' and therefore, triflumizole may be considered as a presumed human reproductive toxicant. We agree that mechanistic data are not available for the developmental effects caused by triflumizole. Although no direct evidence, it is striking that both epoxiconazole and letrazole also induce late resorptions and placental damage in rats. It remains a *possibility* that also for triflumizole, these effects are related to aromatase inhibition. If this would also be the MoA for triflumizole, it cannot be excluded that the effects observed in rats can also occur in humans. Nevertheless, even when the mechanism is different than for epoxiconazole and letrozole, there are no indications that the effects are not relevant for humans. According to the CLP guidance, only 'when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate'. Since there is no such information, classification in Repr. Cat 1B seems more appropriate				

than classification in Repr. 2.
RAC's response
The classification is not based on a comparison with epoxiconazole, but on clear developmental toxicity (reduced number of live fetuses at birth, increased number of dead fetuses at birth, increased number of late resorptions, reduction of fetal weight and increased placental weight) of triflumizole in three studies. Therefore, RAC is of the opinion that classification as Repr. 1B is justified.

Date	Country	Organisation	Type of Organisation	Comment number
29.04.2014	Belgium		MemberState	9
Comment received				
For the reproductive toxicity, several studies are presented in the dossier : • the 2-generation (Tesh et al., 1984), following OECD 416 guidance study reveals an increase of the gestation length for both generation (only significant (2%) for the F0) and a decrease in the conception rate, fertility and % mating in F1 at 12mg/kg bw/d without severe parental modifications. • The teratogenicity study in rats (Nishibe et al., 1983), following OECD guidance 414, shows a reduction in the number of viable foetuses and in foetal body weight together with an increase in the number of late resorptions. The maternal bodyweight is also decreased however the DS indicates that the individual data show late resorption also in dams with a normal bodyweight. • The teratogenicity study in rabbits (Hattori, 1985), following OECD guidance 414, reveals a decrease in foetal weight and placental weight Based on the effects of resorption in the absence of other toxic effects (RIGHT?), we support the classification as category 1B.				
Dossier Submitter's Response				
The increased resorption in the rat was observed in the presence of limited maternal toxicity (reduced body weight gain but not significantly reduced body weight and reduced food consumption). However already a large part of the reduced body weight gain can be explained by the reduced number of foetusses and their lower average body weight. Therefore, it is considered unlikely that these developmental effects are secondary to the maternal toxicity.				
RAC's response				
The opinion is noted and supported by RAC.				

OTHER HAZARDS AND ENDPOINTS – Acute Toxicity

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	Spain		MemberState	10
Comment received				
p. 28 Summary and discussion of acute toxicity Acute oral toxicity The Spanish CA supports the proposed classification of triflumizole as Acute Tox. 4, H302				

(Harmful if swallowed) under Regulation (EC) 1272/2008 (limits LD50 = 300 - 2000 mg/kg bw); and as Xn; R22 (Harmful if swallowed) under Directive 67/548/EC (limits LD50 200 - 2000 mg/kg bw). This classification is based on the results obtained in an acute oral toxicity study in rats (Nishibe et al. 1983a): LD50 = 1057 mg/kg bw.
Dossier Submitter's Response
Thank you for the support.
RAC's response
RAC is of the opinion that triflumizole warrants classification as Acute Tox. 4, H302 (Harmful if swallowed) as supported by Spain.

Date	Country	Organisation	Type of Organisation	Comment number
29.04.2014	Belgium		MemberState	11
Comment received				
We support the classification for the acute toxicity in category 4 (H302) based on : • The study in rats, following the guidance OECD 401(Nishibe et al, 1983) , reveals a LD50 of 1057mg/kg bw/d in males and of 1780mg/kg bw/d in females. These results are within the range of the category 4 (>300 and <2000). • The study in mice, following the guidance OECD 401, indicates a LD50 of 2000mg/kg bw/d in males and of 2800 mg/kg bw/d in females (Nishibe et al., 1983). Due to the study in rats, a classification is warranted in category 4 for the acute toxicity via the oral route.				
Dossier Submitter's Response				
Thank you for the support.				
RAC's response				
RAC is of the opinion that triflumizole warrants classification as Acute Tox. 4, H302 (Harmful if swallowed) as supported by Belgium.				

OTHER HAZARDS AND ENDPOINTS – Skin Sensitisation Hazard

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	Spain		MemberState	12
Comment received				
p. 34 Summary and discussion of skin sensitisation The Spanish CA supports the proposed classification of triflumizole as Skin Sens. 1, H317 (May cause an allergic skin reaction) according to Regulation (EC) 1272/2008; and as Xi; R43 (May cause sensitisation by skin contact) according to Directive 67/548/EC. This classification is based on the results obtained in a skin sensitisation study (Maximisation test) where 8 of the 12 tested animals (66.6%) showed a dermal response after challenge, following a 10% w/v intradermal induction (Nishibe et al. 1983g). Taking into account that the available information does not include an evaluation of the skin sensitisation potential of triflumizole after an intradermal induction at ≤1%, no sub-				

categorization can be performed according to Regulation (EC) 1272/2008, and the classification as Skin Sens. 1 is therefore supported.
Dossier Submitter's Response
Thank you for the support.
RAC's response
RAC is of the opinion that triflumizole warrants classification as Skin Sens. 1, H317 (May cause an allergic skin reaction) as supported by Spain.

Date	Country	Organisation	Type of Organisation	Comment number
29.04.2014	Belgium		MemberState	13
Comment received				
We agree with the proposal to classify for the skin sensitisation. The guinea pig maximisation test show 67% of the tested animals with a positive response after 10% intradermal induction. We understand the difficulty to sub-categorise based on the fact that the substance is not tested with an intradermal induction (≤1%) to classify in category 1A. Then, we support the classification in category 1 without sub classification.				
Dossier Submitter's Response				
Thank you for the support.				
RAC's response				
RAC is of the opinion that triflumizole warrants classification as Skin Sens. 1, H317 (May cause an allergic skin reaction) as supported by Belgium.				

OTHER HAZARDS AND ENDPOINTS – Specific Target Organ Toxicity Repeated Exposure

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	Spain		MemberState	14
Comment received				
p. 50 Summary and discussion of repeated dose toxicity The Spanish CA supports the proposed classification of triflumizole as STOT RE Cat. 2, H373 (May cause damage to the liver through prolonged or repeated exposure) according to Regulation (EC) 1272/2008; and the no classification for repeated dose toxicity, following Directive 67/548/EC criteria. This classification is based on the effects in the liver (weight increase and slight fatty metamorphosis) observed following 28 days exposure at dose levels (265 mg/kg bw/day in rats) below the limits for classification according to Regulation (EC) 1272/2008 (300 mg/kg bw/day), and based on an increase in the severity of liver effects at dose levels above the guidance values in longer oral rat studies. It should be noted that the LOAEL of the combined toxicity/carcinogenicity study (Virgo et al, 1984) is set as 14 mg/kg bw/day (males), just above the cut-off value of				

12.5 mg/kg bw/day established for a 2-year study. Dosis-dependant difuse fatty vauolization of hepatocytes was observed in females at 18 mg/kg bw/day after 104 weeks. However, no doses between 4.5 and 18 mg/kg bw/day were evaluated. Therefore these effects could have been observed in case intermediate doses under the cut-off value (12.5 mg/kg bw/day) would have been tested. Although the results of this study are not sufficient to classify the substance as STOT RE Cat. 2, the effects observed at a dose very close to the cut-off value and the lack of intermediate doses tested, supports this classification.
Dossier Submitter's Response
Thank you for the support.However, it should be noted that the classification is merely based on the significant increase in inflammation/necrosis that was found at ≥ 18 mg/kg bw in females, together wih the increased incidence at 4.5 mg/kg bw in females (although not statistically significant) at both 54 and 104 weeks.
RAC's response
The proposal to classify as STOT RE 2, H373 has been considered and is supported by RAC.

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	France		MemberState	15
Comment received				
Repeated dose toxicity p.53: We agree to classify triflumizole as STOT RE Category 2, H373 according to Regulation (EC) No 1272/2008, based on the liver effects observed in the 28-day and 2-years rat studies. The dossier submitter stated that since the observed necrosis was not widespread or severe, the criteria for classification as R48/22 are not fulfilled. Nevertheless, no information about the severity of the necrosis observed in the 2-years rat study was mentioned in the CLH report. Focal inflammation/necrosis occurred in the females at the dose of 4.5 mg/kg bw/d and above in a dose-related manner. This dose level is below the extrapolated guidance value of 6.25 mg/kg bw/d. Therefore, triflumizole could be classified with Xn, R48/22 as a worst-case.				
Dossier Submitter's Response				
At 4.5 mg/kg bw, the increased inflammation/necrosis in females was not statistically significant. It was significant at ≥18 mg/kg bw. Since this is above the extrapolated guidance value of 6.25 mg/kg bw/d, we conclude that classification according to DSD is not required. However, classification according to DSD is no longer harmonised by RAC.				
RAC's response				
The proposal to classify as STOT RE 2, H373 has been considered and is supported by RAC.				

Date	Country	Organisation	Type of Organisation	Comment number
29.04.2014	Belgium		MemberState	16
Comment received				
For the STOT RE classification, some studies are presented in the dossier : • 90 days study in rats : macroscopic modifications in the liver (increase significantly weight), together with clinical chemistry changes (increase significantly BUN, cholesterol,				

total protein albumin) and presence of fatty metamorphosis. However these modifications are observed at 177mg/kg bw/d in males and 218 mg/kg bw/d in females (outside the range of the category the classification 100mg/kg bw/d) • 28 days study in rats : the liver weight is also increased significantly and the clinical chemistry reveals a significant modification of the cholesterol, total protein and albumin rate at 265mg/kg bw/d in males and 309mg/kg bw/d. A fatty metamorphose is also observed in all tested animals. For males, the result is within the range of the category 2 (≤300mg/kg bw/d)). • 104 weeks in rats : Modifications on the liver are also described : a significant increase of the weight occurring together with microscopic changes - focal inflammation and necrosis (12 males at 3.5mg/kg bw/d and 29 females at 4.5 mg/kg bw/d,out of 70 tested animals/sex), basophilic and eosinophilic foci, … However, the focal inflammation and necrosis is also observed in the control group (13 males and 19 females). Based on these studies, we have some doubts about the need to classify in STOT RE category 2. There are significant effects related to the increased weight, and the changes in the clinical chemistry, however, the liver specific enzymes are not modified. And for the inflammation and necrosis, the control group have already a high rate of positive response. We have doubts about the relevance of this study due to the high response of the control group.
Dossier Submitter's Response
Despite the control group having a relatively high incidence of inflammation/necrosis, it is significantly increased in females at a dose ≥18 mg/kg bw. Although this is slightly above the guidance levels for classification as STOT-RE2, we feel that, considering the severity of an effect as necrosis, together with the effects observed in the 28 day study there is enough reason for classification in STOT-RE2, although we agree this may be a borderline case.
RAC's response
The arguments to not classify as STOT RE 2, H373 has been considered; however, the proposed classification is mostly based on effects of triflumizole observed in a 28-day repeated dose toxicity study, which meets the CLP criteria for classification as STOT RE 2, taking into account the significance and severity of toxic effects occurring at the dose level of oral exposure below or very close to the respective guidance values for that category (300mg/kg bw/d). It is also taking into account that adverse effects in the liver of rats in a 2-year study were found at doses of 14 – 18 mg/kg bw/d, which are very close to the extrapolated guidance value of 12.5 mg/kg bw/d. It is also considered that closer spacing of doses in the 90-day repeated toxicity studies in rats and mice, and using doses just below respective guidance values could reveal adverse effects of triflumizole meeting the classification criteria of STOT RE 2.

OTHER HAZARDS AND ENDPOINTS – Hazardous to the Aquatic Environment

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	Germany		MemberState	17
Comment received				
Page 89 Table 31: summary of relevant information on aquatic toxicity (for triflumizole): There is a minor addition in the results of toxicity to Pseudokirchneriella subcapitata (Manson, 2002c) EbC50 (72h) = 0.858 mg/L and NOEbC (72h) = 0.064 mg/L mean measured.				
Dossier Submitter's Response				

Noted, thank you for the addition. For your information; CLP uses growth as an endpoint (please refer to the Guidance on the Application of the CLP Criteria, Version 4, November 2013) and also this does not change the classification. (*Pseudokirchneriella subcapitata* is formerly known as *Selenastrum capricornutum*)
RAC's response
Noted, thank you for the addition. We agree with the Dossier Submitter's response.

Date	Country	Organisation	Type of Organisation	Comment number
30.04.2014	France		MemberState	18
Comment received				
Environmental hazards We agree with the classification proposal regarding environmental hazard. For the acute and chronic M factors, we also agree with the proposed values.				
Dossier Submitter's Response				
Noted, thank you for the support.				
RAC's response				
Noted.				

Date	Country	Organisation	Type of Organisation	Comment number
29.04.2014	Belgium		MemberState	19
Comment received				
Based on the current available results of the aquatic toxicity tests in the CLH report , the most sensitive species is fish with 96h LC50 = 0.57mg/l (nom.) and a 35dNOEC= 0.044mg/l (nom)). The substance is considered not rapidly degradable. Following the classification criteria of the regulation 1272/2008 it is justified to classify Triflumizole as Aquatic acute 1, H400 and Aquatic chronic 1, H410. However we have some concerns about the determination of the results given and thus the impact on the M-factor. Please find underneath general and specific comments : Comment 1: Please give the references for all aquatic toxicity studies, either in the summary tables or either in the description of the study. Comment 2: Triflumizole is a surface active substance. The results of the aquatic toxicity studies should be considered with care as surfactants can form dispersions or emulsions in which the bioavailablity is difficult to ascertain, even with careful solution preparation. Micelle formation can result in an overestimation of the bioavailable fraction even when "solutions" are apparently formed. Comment 3: In the aquatic toxicity studies on Triflumizole a non-homogeneous dispersion was formed with either un-dissolved material precipitating to the bottom of the test vessel or either forming particles in the dispersion. In general, we are of the opinion that for such substance, the filtered solution gives a more appropriate picture on bioavailability. If the test concentration deviates more than 20% from the nominal concentration, which is merely				

the case for the filtered solutions, measured concentrations should be used.

Comment 4: Based on the available aquatic toxicity results we agree that fish are the most sensitive species.
In the key study for acute toxicity however no analytical analysis was performed and this undermines the validity of the test.
The result of the second acute fish test is of the same order of magnitude than the key study but the results are based on the unfiltered solution and nominal concentrations.
Furthermore recovery is only mentioned for the unfiltered concentration of 2.5mg/l.. Is the recovery for the lower concentrations known?
In the short toxicity study in fish, performed following OECD 203, following effects were seen : increased cough frequency, swimming at different positions in the test vessel, abnormal swimming, ….Therefor we are of the opinion that physical effects cannot be excluded because the substance is a surfactant and has the potential to adsorb to organic matter (Koc = 2764L/kg).

Comment 5: In the Early life stage test with Triflumizole the measured concentrations were much lower than the concentrations measured on previous days. If the analysis on day 35 was not taken into account, the mean recovery varied between 84 and 91%, nevertheless a 35d NOEC is given. OECD guideline 210 (Early life stage) recommends a test duration for Pimephales promelas of 32 days from start of test (or 28 days post-hatch). Maybe it would be, in line with this OECD guideline, more appropriate to determine the NOEC after 32d.

Dossier Submitter's Response

Thank you for your comments, here is our response:
(DAR page numbers refer to DAR Triflumizole 7 V3 B9)

Comment 1: The summaries included in the dossier are partly copied from the DAR, its addenda and assessment reports. We did not evaluate the studies themselves and rely on the DAR, its addenda and assessment reports. References to the individual studies are included in the relevant tables a for more details please refer to the DAR and its addenda.

Comment 2: $\gamma \approx 50$ mN/m at 20C (ref: DAR) As $\gamma<60$ nM/m problems are indeed expected with emulsion formation, which may lead to underestimation of the toxicity (because of reduced bioavailability). However, C&L is based on current knowledge and available data and we cannot assign a larger safety factor based on this (although very valid) suspection.

Comment 3:
Considering precipitation, dispersion and the use of nominal [C]:

- Cannot find any information in the tests about precipitation (one in Fish study Manson 2002a -DAR p.339- , but they did not use this particular [C] where precipitation occured).
- Other studies do report that measured [C] ≥80% of the nominal [C] so for these studies it is justified that they use nominal [C] to calculate the EC50 values. (Studies: DAR Acute Tox. Invertebrate p.11, Acute Tox. Algae p.14, Chronic Tox. Invertebrate p.16)

We do agree with the MS that it would be better to use filtered solutions for toxicity testing. Here, unfiltered solutions, dispersions, are tested, which may mean that the actual bioavailable [C] that caused the observed toxicity is lower than the measured [C]. This leads to an underestimation of the toxicity. However, we have to base the C&L on the available data and cannot change the classification or M factor, as we cannot predict how much lower exactly the EC50 value(s) would be.

Comment 4:
The first study (key study) is indeed nominal.
Second study → yes recovery is known, this is specified DAR p.339 Table B.9.2.1.3a.
Indeed we would also prefer that the LC50 is based on the measured concentrations, not on the nominal ones. However we have to base the C&L on the available data.
To make a very rough estimation; the LC50=0.869 mg/L, lowest recoveries are around 40%. So 40% x 0.869 = 0.35 mg/L, this is still in the same order of magnitude and therefore, this will not change the classification or M factor.
Furthermore, we do acknowledge again the problem of testing unfiltered solutions, as mentioned in the previous comment 3. However, we have to base the C&L on the available data and cannot change the classification or M factor, as we cannot predict how much lower exactly the EC50 value(s) would be.
Also, three trophic levels are tested and not algae, but fish are the most sensitive species for this compound. Therefore, we are probably on the safe side with the EC50 values for algae, although not based on filtered test concentrations.

In the third study on fish indeed physical adverse effects were observed at 0.18 mg/L. As this falls within the 0.1-1 mg/L range this still corresponds to an M factor of 1.

Finally, sorry but we do not understand the relevance of the last argument: "physical effects cannot be excluded because the substance is a surfactant and has the potential to adsorb to organic matter (Koc = 2764L/kg)."
All compounds with higher Koc have the potential to sorb to organic matter and how does this specifically enlarge the potential of physical adverse effects?

Comment 5: (DAR p. 348)
We agree with the MS that the NOEC should be based on 32 days [C] (either nominal or measured, latter preferred). However, we do not have the original study or data so we cannot re-calculate the NOEC. We have to base the C&L on the available data.

RAC's response

Comment 1: Although in the CLH report the reference to the DAR information is clear, the reporting of references to the individual studies could have increased the transparency of the CLH report.

Comment 2: We agree with the MS considerations. We note that for surfactants, the general approach is to compare toxic effect concentrations with the critical micelle concentration (CMC) for a substance in water rather than with its water solubility limit. If the E(L)C50 or NOEC(L) is below the CMC then the CLP criteria can be applied directly to the data. If the substance is not toxic at the CMC, the CMC may be used as a NOEC. If a test has been conducted at concentrations above the CMC and shows effects, the effect concentration should be set as the CMC as a precautionary worst case, unless it is clear that physical effects have occurred.

However, we noticed that in the CLH report it is just stated that undissolved material was revealed at concentrations higher than effect concentrations (5,0 and 10,0 mg/l in the 2° short-term study on fish). It can therefore be assumed that physical effects of undissolved material in the two highest concentrations have not influenced the calculated LC50 value.
Moreover, in the DAR the RMS considered the studies as reliable, even if the results are based on non-filtered concentration.

Comment 3: We agree with the MS that it would be better to use filtered solutions for toxicity testing. In the CLH report, where the measured concentrations of filtred solution are

reported, these are always below 80% of nominal concentrations. However, in the DAR the RMS considered that all the studies, where unfiltered solutions and dispersions are tested, are acceptable.

Comment 4: The MS observations are reasonable, but RAC agrees with the DS that these do not change the classification or M-factor.
Moreover, concerning the argument on physical effects in a short toxicity study in fish, we highlight that in the DAR it is stated that physical effects of undissolved material have not influenced the calculated LC50 value, because the undissolved material was observed just at the two highest concentrations far above the LC50 (see response to comment 2).

Comment 5: We agree with the MS, but in the DAR the RMS assumed that deviations from the expected concentrations were restricted to a small period of time and have not influenced the results of the test. The study duration was 35 days from the start of the test and 30 days post-hatch, that is only a two-day deviation for the post-hatch test duration as defined in the OECD guideline 210, and therefore the RMS assumption could be plausible.

ATTACHMENTS RECEIVED

The following were submitted by Nisso Soda Co., Ltd on 30.04.2014. *[Please refer to comment number 7]*

1. Albrecht ED, Aberdeen GW, Pepe GJ (2000): The role of estrogen in the maintenance of primate pregnancy. Am. J. Obstet. Gynecol. 182(2) pp. 432-438 *[Filename: 2 Albrecht ED Am j Obstet. Gynecol. 2000]*

2. Chibbar R, Hobkirk R, Mitchell BF (1986): Sulfohydrolase activity for estrone sulfate and dehydroepiandrosterone sulfate in human fetal membranes and deciduas around the time of parturition. J. Clin. Endocrin. Metab. 62(1) pp. 90-94 *[Filename: 5 Chibbar R (1986)]*

3. France JT (1979): Steroid sulphatase deficiency. J. Steroid Biochem. 11 pp. 647- 651 *[Filename: 6 France JT J. Steroid Biochem._1979]*

4. Glutek SM, Hobkirk R (1990): Estrogen sulfatase and steroid sulfatase activities in intrauterine tissues of the pregnant guinea pig. J. Steroid Biochem. Mol. Biol. 37(5) pp. 707-715 *[Filename: 7 Glutec SM J. Steroid Biochem. Molec1990]*

5. Jackson JA, Albrecht ED (1986): Estrogen regulates placental androstenedione production during rat pregnancy. Endocrinology 119(3) pp. 1052-1057 *[Filename: 8 Jackson JA (1986)]*

6. Keyes PL, Yuh KC, Miller JB (1979): Estrogen action in the corpus luteum. Adv. Exp. Med. Biol. 112 pp. 447 – 463 *[Filename: 10 Keyes PL (1979)]*

7. Kragie L, Turner SD, Patten CJ, Crespi CL, Stresser DM (2002): Assessing pregnancy risks of azole fungicides using a high throughput aromatase inhibition assay. Endocr. Res. 28(3) pp. 129-140 *[Filename: 11 Kragie L (2002)]*

8. Mason JI, Murry BA, Olcott M, Sheets JJ (1985): Imidazole antimycotics: inhibitors of steroid aromatase. Biochem. Pharmacol. 34 pp. 1087-1092 *[Filename: 12 Mason JI Biochm. Pharmacol. 1985]*

9. Mason JI, Carr BR, Murry BA (1987): Imidazole antimycotics: selective inhibitors of steroid aromatisation and progesterone hydroxylation. Steroids 50(1-3) pp. 179-189 *[Filename: 13 Mason JI Steroids 1987]*

10. Moreno MCR, Fussell KC, Gröters S, Schneider S, Strauss V, Stinchcombe S,Feggert I, Veras M, van Ravenzwaay B (2013): Epoxiconazole –induced degeneration in rat placenta and the effects of estradiol supplementation. Birth Defects Research (Part B) 98(3) pp. 208-221 *[Filename: 15 Moreno MCR Birth Defects Researc 2013]*

11. Schneider S, Hofmann T, Stinchcombe S, Moreno MCR, Fegert I, Gröters S, Fabian E, Thiaener J, Fussel KC, van Ravenzwaay B (2013): Species differences in developmental toxicity of Epoxiconazole and its relevance to humans. Birth Defects Research (Part B) 98(3) pp. 230-246 *[Filename: 17 Schneider S Birth defects Researc 2013]*

12. Stinchcombe S, Schneider S, Fegert I, Moreno MCR, Strauss V, Gröters S, Fabian E, Fussel KC, Pigott GH, van Ravenzwaay B (2013): Effects of estrogen coadministration on Epoxiconazole toxicity in rats. Birth Defects Research (Part B) 98(3) pp. 247-259 *[Filename: 19 Stinchcombe S irth Defects Researc 2013]*

13. Taxvig C, Vingard AM, Hass U, Axelstad M, Metzdorff S, Nelleman C (2008): Endocrine disrupting properties in vivo of widely used azole fungicides.Int. J. Androl. 31(2) pp. 170-177 *[Filename: 22 Taxvig C (2008)]*

14. Trösken ER, Scholz K, Lutz RW, Völkel W, Zarn JA, Lutz WK (2004): Comparative assessment of the inhibition of recombinant human CYP19 (aromatase) by azoles used in agriculture and as drugs for humans. Endocr. Res. 30(3) pp.387-394 *[Filename: 25 Trosken ER (2004)]*

Annex I: JMPR Summary of Gotoh, 1986

In a developmental toxicity study in rats, groups of 24 pregnant female Sprague-Dawley (Crj:CD) rats were treated with triflumizole (purity 98.3%; lot no. TK4121) by gavage at a dose level of 0, 3, 7 or 35 mg/kg bw per day. The study was performed partly in accordance with OECD Test Guideline 414. Rats were dosed from GD 6 up to GD 16, but a caesarean section was not performed until GD 20. OECD guidelines state that doses should be administered daily from implantation to the day before caesarean section. Females were examined daily throughout the study for mortality and clinical signs. Body weight and feed consumption were recorded daily on GDs 0–20. Water intake was measured on GDs 1, 6, 11, 16 and 20. On GD 20, females were killed and subjected to a caesarean section and gross necropsy. The uterus was removed and opened, and fetuses were removed. Numbers of viable fetuses, early and late resorptions, total implantations and number of corpora lutea were recorded. Uteri from apparently non-pregnant females were stained with ammonium sulfide solution for confirmation of pregnancy status. All viable fetuses were weighed, sexed and examined for external malformations and variations. The total numbers of fetuses examined (number of litters) were 339 (24), 343 (23), 342 (24) and 317 (24) for the 0, 3, 7 and 35 mg/kg bw per day groups, respectively. Approximately half of the fetuses were placed in Bouin's fixative and examined for visceral malformations and variations, and the remaining half of the fetuses were fixed in ethanol and stained for examination for skeletal malformations and variations.

Maternal toxicity was evident at 35 mg/kg bw per day. Body weight gain was reduced compared with controls over GDs 17–18 and over the dosing interval (−18% and −15%, respectively; $P < 0.05$). The reduction in mean body weight gain was accompanied by reductions in feed consumption on GD 7 and daily over GDs 12–19, ranging from −9% to −16% of control values. No statistically significant differences were noted in absolute body weight or water intake. Changes in organ weights were not definitive of adverse effects of treatment. The mean absolute right adrenal weight was significantly decreased, but was not accompanied by changes in relative weight. The mean left ovary weight relative to body weight was significantly increased, but the right relative ovary weight was comparable to that of controls. The placental weight was significantly increased compared with controls (+45%; $P < 0.01$). Gross necropsy did not reveal any adverse effects of treatment. No adverse effects of treatment were observed in females treated with 3 or 7 mg/kg bw per day. Evidence of developmental toxicity was seen at 35 mg/kg bw per day. The incidence of late resorptions/dead fetuses was significantly ($P < 0.05$) increased (17 versus 0 for controls; 4.8% versus 0% for controls). Although the number of viable fetuses was slightly reduced compared with controls (13.2 versus 14.1 for controls), this reduction did not attain statistical significance. Fetal weight was not affected (Table 34). No statistically significant, treatment-related external, visceral or skeletal malformations or variations were noted.

Table 34. Fetal toxicity in a developmental toxicity study in rats

Table 34. Fetal toxicity in a developmental toxicity study in rats

	0 mg/kg bw per day	3 mg/kg bw per day	7 mg/kg bw per day	35 mg/kg bw per day
Litter response				
Live fetuses/ pregnant female	14.1	14.9	14.3	13.2 (−6%)
Dead or resorbed fetuses	23	13 (−43%)	24	35 (+52%)
- Early deaths	23	12	21	18
- Late deaths	0	1	3	17*

*: $P < 0.05$; ** $P: < 0.01$
Source: Gotoh (1986)

The maternal toxicity NOAEL was 7 mg/kg bw per day, based on reductions in body weight gain and feed consumption and increased placental weights at 35 mg/kg bw per day. The embryo/fetal toxicity NOAEL was 7 mg/kg bw per day, based on increased numbers of late resorptions/dead fetuses at 35 mg/kg bw per day (Gotoh, 1986).