Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

VKM Report 2015:10

Risk assessment of the metabolite M44 of bixafen, one of the active substances in Aviator Xpro EC 225

Opinion of the Panel on Plant Protection Products of the Norwegian Scientific Committee for Food Safety

Report from the Norwegian Scientific Committee for Food Safety (VKM) 2015:10
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11.06.2015

ISBN: 978-82-8259-165-2
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Cover photo: iStock Photo

Suggested citation: VKM (2015). Risk assessment of the metabolite M44 of bixafen, one of the active substances in Aviator Xpro EC 225. Opinion of the Panel on Plant Protection Products of the Norwegian Scientific Committee for Food Safety. VKM Report 2015:10, ISBN: 978-82-8259-165-2, Oslo, Norway. Available online: www.vkm.no

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Assessed and approved

The opinion has been assessed and approved by Panel on Plant Protection Products of VKM. Members of the panel are:

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Acknowledgment

Project manager from the VKM secretariat has been Edgar Rivedal. Jan Ludvig Lyche, Hubert Dirven, Marit Låg and Asbjørn M Nilsen are acknowledged for their valuable work on this opinion

Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM

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Summary

Aviator Xpro EC 225 containing the active substance bixafen was assessed by VKM in spring 2013, and it was concluded that the metabolite M44 has potential for groundwater contamination. Furthermore, VKM assessed in late 2013 the relevance of this metabolite in accordance with the EU guidance document on metabolites in groundwater, and concluded that the malformations observed in rabbits exposed to the metabolite should be considered treatment related. VKM also concluded that the data presented to evaluate the possible genotoxic properties of the metabolite was insufficient to reach a conclusion. Based on this, the Norwegian Food Safety Authority rejected the approval of Aviator Xpro EC 225.

The applicant has now submitted results from an *in vivo* study to strengthen the basis for assessment of genotoxic properties, and also submitted new historical controls in relation to the experimental studies on foetal developmental effects in rabbits. The VKM Panel on Plant Protection Products has discussed the questions raised by The Norwegian Food Safety Authority on the basis of the new data, and has the following opinion:

On the assessment of genotoxic properties of the M44 metabolite of bixafen, one of the active ingredients of Aviator Xpro EC 225

It is the view of VKM Panel on Plant Protection Products that the new *in vivo* mouse micronucleus study, supplemented together with a separate study demonstrating bioavailability, overrides the results of the *in vitro* clastogenicity studies. Taken together, it is the opinion of VKM that under the conditions studied, M44 should be considered as non-genotoxic.

On the assessment of the relevance of the foetal malformations in M44 exposed animals

VKMs Panel on Plant Protection products has assessed the arguments and new historical control data presented by the applicant, intended to show that metabolite M44 is not teratogenic. It is however the opinion of the Panel that the arguments and the new historical data provided by the applicant do not alter the panel's previous conclusion; that the malformations observed in rabbits exposed to the metabolite M44 should be considered treatment related.

Key words: VKM, risk assessment, Norwegian Scientific Committee for Food Safety, pesticide, ground water, Aviator, bixafen, metabolite, M44

Sammendrag på norsk

Aviator Xpro EC 225 med det virksomme stoffet bixafen ble vurdert av VKM våren 2013, og det ble konkludert med at metabolitten M44 har potensiale for grunnvanns-forurensning. Senere samme år konkluderte VKM med at misdannelser observert hos kanin bør ansees å være knyttet eksponering for metabolitten. VKM var også av den oppfatning at dataene for å vurdere om metabolitten kan skade arvematerialet var utilstrekkelige. Basert på dette avslo Mattilsynet å godkjenne Aviator Xpro EC 225 for bruk i Norge.

Søker har nå levert nye resultater fra en *in vivo* studie for øke grunnlaget for å kunne vurdere om metabolitten kan ha arvestoffskadelige egenskaper, og dessuten nye historiske kontroller til å supplere studiene av fostereffekter i kanin. VKMs Faggruppe for plantevernmidler har diskutert spørsmålene som reises av Mattilsynet på bakgrunn av søkers klage, og har følgende konklusjon:

Vurdering av arvestoffskadelige egenskaper hos metabolitten M44

VKMs Faggruppe for plantevernmidler er av den oppfatning at det ny-innsendte forsøket, et i*n vivo* mus mikrokjerneforsøk, sammen med måling av stoffets biotilgjengelighet, gjør at M44 ikke anses å være arvestoff-skadelig.

Vurdering av misdannelser i kaniner eksponert for M44

VKMs Faggruppe for plantevernmidler har vurdert søkers argumenter og nye historiske kontrolldata, som er ment å vise at metabolitten M44 er ikke har fosterskadelige effekter. VKM er imidlertid av den oppfatning at argumentene og de nye historiske dataene fra søker ikke endrer faggruppens tidligere konklusjon; at foster-misdannelser observert hos kanin eksponert for metabolitten M44 synes å være resultat av eksponeringen for M44.

Abbreviations

DMSO	dimethyl sulfoxide
In vivo	Experiment on living organisms
In vitro	"In glass" – experiment outside an organism – in test tube
NOAEL	No Observed Adverse Effect Level
VKM	Norwegian Scientific Committee for Food Safety

Background as provided by the Norwegian Food Safety Authority

The new plant protection product Aviator Xpro EC 225 containing the new active substance bixafen was assessed by the VKM in spring 2013 (VKM, 2013a). In the VKM assessment of environmental fate and behavior, it was concluded that a metabolite coded M44 has potential for groundwater contamination in concentration above 0.1 µg/L. The Norwegian Food Safety Authority therefore asked the VKM to assess the relevance of this metabolite in accordance to the "Guidance Document on the Assessment of the Relevance of Metabolites in Groundwater of Substances Regulated Under Council Directive 91/414/EEC" (European Commission, 2003). VKM assessed the relevance of this metabolite in December 2013, and concluded that the "misshapen interparietal bone" and the "severely malformed vertebral column and/or ribs" should be considered treatment related (VKM, 2013b). Based on the VKM conclusion, the Norwegian Food Safety Authority rejected the approval of Aviator Xpro EC 225.

In the VKM relevance assessment of the bixafen groundwater metabolite M44 in December 2013, VKM concluded that the presented data from *in vitro* experiments did not provide the necessary assurance that metabolite M44 was negative for genotoxic activity (VKM, 2013b). No data for *in vivo* testing of the metabolite was presented for the VKM evaluation in 2013. However, the applicant has later submitted an *in vivo* genotoxicity study to confirm that the metabolite M44 is negative in terms of genotoxicity.

Terms of reference as provided by the Norwegian Food Safety Authority

The applicant has filed an appeal against the Norwegian Food Safety Authority's decision to reject the approval of Aviator Xpro EC 225. The applicant has provided new historical control data for the above-mentioned findings of foetal malformations. The Norwegian Food Safety Authority asks VKM to assess whether the arguments and the new historical control data presented by the applicant give reason to revoke the decision on metabolite M44 with regards to its ability to induce foetal malformations, and whether the newly submitted genotoxicity study provides the necessary assurance for concluding on the non-genotoxicity of metabolite M44.

Assessment

1 Introduction

Aviator Xpro EC 225 containing the active substance bixafen was assessed by VKM in 2013 (VKM, 2013a). In this assessment it was concluded that the metabolite M44 had properties which suggested a potential for groundwater contamination above the concentration of 0.1 µg/L. Based on this, The Norwegian Food Safety Authority requested VKM to assess this metabolite in accordance with the "Guidance Document on the Assessment of the Relevance of Metabolites in Groundwater of Substances Regulated under Council Directive 91/414/EEC" (Sanco/221/2000-rev.10-final, 25 February 2003) (European Commission, 2003). This was done in a meeting in December 2013, and VKM concluded that the finding of "misshapen interparietal bone" and "severely malformed vertebral column and/or ribs" should be considered treatment related (VKM, 2013b). Based on this conclusion, the Norwegian Food Safety Authority did not approve the application for registration of Aviator Xpro EC 225 in Norway.

Following the rejection, the applicant filed an appeal against the Norwegian Food Safety Authority's decision not to approve the registration of Aviator Xpro EC 225 for use in Norway. In the appeal the applicant included additional historical control data linked to the reported findings of malformations in the reproduction studies. The Norwegian Food Safety Authority has requested an opinion from VKM whether the new historical control data and the arguments presented by the applicant are sufficient to alter the previous conclusion regarding the above mentioned foetal malformations observed in animal experiments following exposure to the metabolite M44.

VKM's assessment is based on the Norwegian Food Safety Authority's evaluation of the documentation submitted by the applicant.

2 Hazard identification and characterisation

2.1 Summary of VKMs view on adverse test findings

VKM had in its previous report the following conclusions regarding the presented test results for bixafen and the metabolite M44:

2.1.1 Bixafen

The liver effects and effects on coagulating parameters were considered adverse and of relevance to humans.

The thyroid follicular cell tumours reported in female rats were not considered sufficient to suggest that bixafen had a tumour inducing potential.

The presented data was not considered sufficient to exclude that the observed reduction in pup weight and increased number of stillborn pups in the F1 and F2 generation were induced by bixafen.

The reported pup developmental effects were considered treatment related, and not regarded as secondary effects to maternal toxicity.

2.1.2 Metabolite M44

The *in vitro* experiments on genotoxicity were not considered to provide sufficient assurance that the bixafen metabolite M44 was negative for genotoxic activity.

The foetal malformations («misshapen intraparietal bone» and «severely malformed vertebral columns and/or ribs») were considered treatment related.

2.2 Re-assessment of the relevance of the foetal malformations in M44 exposed animals

The applicant has appealed the decision and has presented arguments and new historical control data, to show that metabolite M44 is not teratogenic.

2.2.1 The applicant's arguments for why the metabolite M44 should not be regarded as teratogenic

2.2.1.1 «Misshapen interparietal bone»

The applicant argues that «misshapen interparietal bone» has a high natural occurrence in the rabbit line and shows that 10 of 38 skeletal malformations were «misshapen interparietal bone» in the historical control data. The applicant claims that this deformity occurs randomly because of the comparable incidence in both control and dose groups in other teratology studies at the same lab using the same rabbit line.

2.2.1.2 "Severely malformed vertebral column and/or ribs"

The applicant disagrees with VKMs statement that "the lack of a dose-response relationship for this effect could be masked by the increased maternal mortality at the highest dose level." The applicant argues that there is no relationship between maternal toxicity and "severely malformed vertebral column and/or ribs", and that the absence of such a relationship makes it very unlikely that the increased maternal mortality will mask a dose-response relationship. Furthermore, the number of litters with live foetuses is comparable in middle (17 litters) and highest (18 litters) dose group, suggesting that the occurrence of the abnormality should increase in the highest dose group despite increased maternal mortality. The applicant has also summarized a number of authorities that have considered the increased incidence of "severely malformed vertebral columns and / or ribs" in the middle dose group as random and not treatment related, because of lack of dose-response.

2.2.2 VKM's opinion on the observed effects in rabbit foetuses following exposure of the mother animal to M44

Despite the comparable number of litters in middle and highest dose group, the number of foetuses is still lower in the highest dose group. Furthermore, despite increased number of abortions in the highest dose group, the aborted foetuses were not examined for skeletal malformations, and thus it is not possible to assess whether abortions were associated with skeletal malformations in the highest dose group. However, the Canadian Pest Management Regulatory Agency (PMRA) established a developmental NOAEL of 300 mg / kg bw / day from this study, based on the increase in the number of abortions (http://www.hc-sc.gc.ca/ahc-asc/branch-dirgen/pmra-arla/index-eng.php). Likewise, the increase in the

number of abortions is also assumed to be treatment-related by the manufacturer but related to the general toxicity of the females in the highest dose group.

2.2.3 VKM's opinion on the observed effect of M44 on rabbit foetuses in light of the new historical control data

2.2.3.1 The use of historical control data

Due to limited numbers of unexposed control animals in each experimental study, and often only one or two experiments performed for each pesticide, values for untreated animals from other experiments (so-called historical controls) have been used to strengthen the findings in individual experiments. A number of requirements must however be fulfilled in order to use other data than the data from the actual test experiment of the relevant pesticide

2.2.3.2 Quality of historical control data

There are a number of requirements for the quality and use of historical control, for example illustrated by the British Health and Safety Excecutive (HSE, 2004), and in in Annex 1, section 5.5 or 5.6 of Directive 94/79/EC (European Commission, 1994).

According to the guidelines, historical control data should be from studies that are identical or closely related to the study under consideration. The historical control data should not be more than +/- two years compared with the current study, and studies must partly be performed with the same species and animal line, at the same lab and under comparable condition.

The historical data should also be presented on a study by study basis, or at least should detailed data on the variation of the historical controls be presented, such as the range of values, the mean, median and, if applicable, standard deviation.

The submitted historical control data should be from studies that are identical or closely related to the study under consideration in terms of:

1. Species, strain and supplier.

2. Test facility.

3. Housing conditions (single or multiple) and diet.

4. Survival rates (especially for carcinogenicity studies).

5. Date of performance - typically +/- two years. Values and incidences vary with time and it is essential to have information on studies performed within a relatively short time period. Although retrospective data will only be available at the time of writing a study report, additional studies will normally be available by the time a dossier is submitted. The

more recent data are particularly relevant and should be submitted. Periods greater than +/- 2 years might be justifiable if the test facility does not perform the particular study type routinely.

6. Assessment criteria (e.g. terminology / definitions for histopathology and developmental findings or clinical chemistry assay methodology).

7. Age or weight of animals at initiation of study and / or the time of investigation (especially for short-term studies).

8. Group size.

9. Dose route. The submission must provide adequate supporting information to permit independent confirmation of the applicability of the historical control data, based on the above criteria. There should also be a statement that the data cover all appropriate studies performed by the test facility during the period under consideration, and have not been selected in any way.

There is no guarantee that historical control data that meet the criteria defined above will be used in an assessment. For example where pre-dosing data, or incidences in lower dose groups, are consistent with the concurrent controls the assumption would be that the concurrent controls are truly representative of the background levels pertaining at the time of the study.

2.2.3.3 "Old" and "New" historical control

The teratology study in rabbits with the metabolite M44 performed in 2008 was the first study using New Zealand White Rabbits in BASF Toxicological Laboratory. The applicant claims that the previously submitted historical control data should be replaced by new data because the old historical controls were based on four studies performed 7 and 9 years earlier. The applicant argues that the new submitted data is more relevant because they were performed in a period closer to the current study and because the same rabbit line (NZW rabbits) was used. However, the new historical control data does not fulfil all the requirements of the guidelines because some of the studies are up to 8 years younger than the M44 teratology study, and in June 2013 there was a shift in the supply of laboratory animals.

The new historical control data presented by the applicant is indicated in Table 2.2.3.3-1 and 2.2.3.3-2. The tables summarizes the incidence of malformations in the M44 teratology study, as well as the occurrence of these malformations in the "new" and "old" historical control data. As indicated in the tables, the occurrence of "misshapen interparietal bone" in teratology metabolite M44 of 1.9%, the occurrence of this malformation is beyond even the new historical control data.

Table 2.2.3.3-1. Incidence of «misshapen interparietal bone». Study with M44 and historical controls. *: statistically different from control.

Year	Fetal incidence (n/tot)				Affected fetuses/litter (%)			
	control	Low dose	Mid dose	High dose	control	Low dose	Mid dose	High dose
2009	1/351	1/153	1/152	3/138	0.2	0.6	0.4	1.9*
2009	3/184				1.2			
2009	2/156				0.6			
2010	1/251				0.6			
2010	0/189				0			
2011	2/232				1.4			
2012	2/150				0.9			
2012	0/214				0			
2012	0/125				0			
2013	0/194				0			
2013	0/154				0			
2013	0/186				0			
2013	0/126				0			
2014	0/234				0			
Historical controls	10/2395							

Table 2.2.3.3-2. Incidence of "severely malformed vertebral column and/or ribs". Study with M44 and historical controls. *: statistically different from control.

Year	Fetal incidence (n/tot)				Affected fetuses/litter (%)			
	control	Low dose	Mid dose	High dose	control	Low dose	Mid dose	High dose
2009	0/351	0/153	4/152	1/138	0.0	0.0	2.9*	0
2009	1/184				0.4			
2009	1/156				0.4			
2010	0/251				0			
2010	0/189				0			
2011	0/232				0			
2012	0/150				0			
2012	0/214				0			
2012	0/125				0			
2013	0/194				0			
2013	1/154				0.5			
2013	0/186				0			
2013	0/126				0			
2014	1/234				0.6			
Historical controls	4/2395							

The incidence of "severely malformed vertebral column and/or ribs" in middle dose group of 2.9% is also outside the new historical control data, while the incidence of this abnormality in the highest dose group no longer is outside historical control data.

The values of 1.4% and 0.6% affected foetuses/litter for "misshapen interparietal bone" and for "severely malformed vertebral column and/or ribs" presented by the applicant as new historical controls. However, these values represent the upper range of study means for the respective finding and not the average over all 13 studies of the historical control.

The new historical control value of 1.4% for "misshapen interparietal bone" is based on data from one study conducted more than two years after the M44 study. Furthermore, the new historical control value of 0.6% for "severely malformed vertebral column and/or ribs" is based on one study conducted in 2014, which is 5 years after the M44 study. The 2014 study was also performed with rabbits from a new supplier. These conditions are not in line with central requirements in the guidelines, which state that performance date for historical control data should not be more than +/- two years, and studies should be performed with the same species, strain and supplier.

2.2.3.4 The procedure for using historical control data

Historical controls may be used to discuss if the higher values observed for treated animals than for controls in the same study may have occurred by co-incidence. There seems to have been a tendency that most emphasis have focused on the single highest value obtained in historical control groups when assessing the effect observed in the exposed animal groups.

It is the opinion of the VKM panel that the implication of historical controls should preferably be assessed using statistical analysis, pending that the historical controls fulfils the quality criteria. An example of such analysis would be to use 2X2 contingency tables in two-sided Fisher Exact Test.

When Fisher Exact Test is used for the present experiments and historical controls, the following results are obtained, as shown in Tables 2.2.3.4-1 and 2.2.3.4-2:

Table 2.2.3.4-1. «Misshapen interparietal bone». Two-sided Fischer exact test

Tested groups	Contingency table	P-value
Control vs low dose	1/351 vs 1/153	0.52
Control vs medium dose	1/351 vs 1/152	0.52
Control vs high dose	1/351 vs 3/138	0.073
Historical controls vs high dose	11/2746 vs 3/13	0.024*
Actual vs historical controls	1/351 vs 10/2395	0.93

Such analysis of the «misshapen interparietal bone» data shows that the finding of 3 affected foetuses in the highest dose is borderline statistically significant (P=0.073) when using the actual control. When including historical controls, the same finding becomes statistically significant (P=0.024)

Table 2.2.3.4-2. "Severely malformed vertebral column and/or ribs". Two-sided Fischer exact test

Tested groups	Contingency table	P-value
Control vs low dose	0/351 vs 0/153	
Control vs medium dose	0/351 vs 4/152	0.0087*
Control vs high dose	0/351 vs 1/138	0.28
Historical controls vs medium dose	4/2746 vs 4/152	<0.0001*
Actual vs historical controls	0/351 vs 4/2395	0.99

The Fischer Exact Test used on the data for "severely malformed vertebral column and/or ribs" shows that the 4 affected foetuses in the middle concentration is statistically increased above control, also without use of the historical controls. Again, the P-value becomes more significant when the historical controls are included in the analysis.

Thus, it is the opinion of VKM's panel on plant protection products that the provision of arguments and new historical controls by the applicant does not alter the previous conclusion; that the "misshapen interparietal bone", and "severely malformed vertebral column and/or ribs" should be considered treatment related.

The applicant argues that there is no relationship between maternal toxicity and "severely malformed vertebral column and/or ribs", and that the absence of such relationship makes it unlikely that the increased maternal mortality will mask a dose-response relationship. However, the applicant does not provide any scientific evidence for these arguments. Thus, the VKM panel maintains its previous statement that "the lack of a dose-response relationship for this effect could be masked by the increased maternal mortality at the highest dose level."

2.3 Re-assessment of the genotoxic potential for M44

2.3.1 Background

For bixafen, the data from a complete battery of *in vitro* and *in vivo* genotoxicity studies was reported, and based on these studies; VKM concluded that bixafen was not considered genotoxic (VKM, 2013a).

The metabolite M44 was tested for mutagenicity in four strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and one strain of *Escherichia coli* (WP2 uvrA) with and without metabolizing S9-mix, and no indication of a response was reported.

M44 was also tested for induction of gene mutations in Chinese hamster CHO cells with and without metabolic S9-mix in two independent experiments. No statistically significant increases in mutant frequency were observed.

M44 was evaluated for clastogenic effects *in vitro* in Chinese hamster V79 cells, with and without metabolizing S9-mix. A mixed response was obtained in four different studies.

Experiment 1: Increased numbers of aberrant metaphase cells with and without S9-mix. The increase with S9-mix was statistically significant.

Experiment 2: Increased numbers of aberrant metaphase cells without S9-mix. The increase at highest dose (1.6 mg/ml) was statistically significant.

Experiment 3 could not be evaluated.

Experiment 4: Statistically significant increase in the number of aberrant metaphase cells with S9-mix.

In the report of 2013 VKM expressed its concern on the use of "historical controls" to override findings in individual experiments and concluded as follows: "The presented data from *in vitro* experiments do not provide the necessary assurance that the bixafen metabolite M44 is negative for genotoxic activity. No data for in vivo testing of the metabolite have been presented. It is therefore the opinion of VKM that the submitted studies are not adequate to evaluate possible health risk of the metabolite M44 in accordance with the relevant Guidance Document."

2.3.2 New data presented

The applicant has now presented data from an in vivo mouse micronucleus test in bone marrow cells with the bixafen metabolite M44. The study is conducted in accordance to the OECD guideline 474 and according to GLP.

The mammalian in vivo micronucleus test is used for the detection of damage by the test item to the chromosomes or the mitotic apparatus of immature erythrocytes (so called polychromatic erythrocytes) in the bone marrow. Groups of 5 male mice were treated with a single oral dose of the bixafen metabolite M44, dissolved in DMSO, at dose levels of 375, 750 and 1500 mg/kg bw. Doses were selected based on a Dose-Range Finding study. The Dose-Range finding study did not demonstrate differences between sexes in toxicity, so testing in a single sex in the main study is justified. DMSO was used as vehicle control and cyclophosphamide and vincrine sulphate were used as positive controls for chromosome damage or damage to the mitotic spindle effects, respectively. The animals were sacrificed

24 hours following exposure. An additional group of animals treated with 1500 mg/kg bw , were sacrificed at 48 hours Bone marrow was prepared from the 2 femora and after staining 2000 polychromatic erythrocytes were evaluated per animal and investigated for micronuclei. The mature erythrocytes (so called normocytes) with and without micronuclei occurring per 2000 polychromatic erythrocytes were also recorded.

In a separate study on the kinetics of the bixafen metabolite M44 in mice, the bioavailability of the test substance in bone marrow after oral administration was demonstrated. Five hours after dosing low levels of M44 and its metabolites were found in plasma, blood cells and bone marrow.

The incidences of micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes in male mice treated with M44 were not statistically significantly different from those found for the vehicle control. The positive control groups differed statistically significantly from the vehicle control group, demonstrating the sensitivity of the system. No relevant inhibition of erythropoiesis determined from the ratio of polychromatic to normochromatic erythrocytes was detected.

These data support the conclusion that, at the doses achieved under the conditions used in this study, the test item M44 did not produce chromosomal damage or damage to the mitotic spindle apparatus in the bone marrow of mice.

It is the opinion of VKM that this *in vivo* study overrides the results of the *in vitro* clastogenicity study. Taken together it is the opinion of VKM that under the conditions studied M44 should be considered as non-genotoxic.

3 Conclusions

On the assessment of genotoxic properties of the M44 metabolite of bixafen, one of the active ingredients of Aviator Xpro EC 225

It is the view of VKM Panel on Plant Protection Products that the new *in vivo* mouse micronucleus study, supplemented together with a separate study demonstrating bioavailability, overrides the results of the *in vitro* clastogenicity studies. Taken together, it is the opinion of VKM that under the conditions studied, M44 should be considered as non-genotoxic.

On the assessment of the relevance of the foetal malformations in M44 exposed animals

VKMs Panel on Plant Protection products has assessed the arguments and new historical control data presented by the applicant, intended to show that metabolite M44 is not teratogenic. It is however the opinion of the Panel that the arguments and the new historical data provided by the applicant do not alter the panel's previous conclusion; that the malformations observed in rabbits exposed to the metabolite M44 should be considered treatment related.

4 References

European Commission. (1994) COMMISSION DIRECTIVE 94/79/EC concerning the placing of plant protection products on the market.

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VKM. (2013a) Risk assessment of the fungicide Aviator Xpro EC 225 with the active substances bixafen and prothioconazole.

VKM. (2013b) Risk assessment of the metabolite M44 of bixafen, an active substance in the fungicide Aviator Xpro EC 225.