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Committee for Medicinal Products for Veterinary Use

## European public MRL assessment report (EPMAR) Diflubenzuron (*Salmonidae*)

On 8 November 2019 the European Commission adopted a Regulation<sup>1</sup> establishing maximum residue limits for diflubenzuron in *Salmonidae*, valid throughout the European Union. These maximum residue limits were based on the favourable opinion and the assessment report adopted by the Committee for Medicinal Products for Veterinary Use.

Diflubenzuron is intended for use in Atlantic salmon for the treatment of sea lice (*Lepeophtheirus salmonis*) infestations, with an intended oral dosage of 3-6 mg diflubenzuron/kg bw/day during 14 consecutive days.

Diflubenzuron was previously assessed by the CVMP and was included in Table 1 of the Annex to Commission Regulation (EU) No. 37/2010 with an MRL in *Salmonidae*.

On 7 May 2014 the European Commission submitted to the European Medicines Agency a request under Article 11 of Regulation (EU) No 470/2009 to issue a new opinion on diflubenzuron taking into account the genotoxic potential of diflubenzuron's metabolite 4-chloroaniline, and the more recent evaluations of diflubenzuron as a pesticide and a biocide, undertaken by the European Food Safety Authority (EFSA)<sup>2</sup>, and coordinated by and the Commission's Joint Research Centre (JRC)<sup>3</sup>, respectively.

In May 2015, the CVMP adopted an opinion recommending that the previous MRL for diflubenzuron should be converted to a provisional MRL while data is sought on formation and depletion of the metabolite in fish muscle.

In March 2017, the Commission clarified that the establishment of a provisional MRL was not considered appropriate in this case and highlighted the EFSA 2015 conclusion<sup>4</sup> relating to the use of diflubenzuron in plant protection products. The Commission invited the CVMP to revise its opinion of May 2015.

Based on the original and complementary data, the Committee for Medicinal Products for Veterinary Use recommended on 15 March 2018 the modification of the maximum residue limit for diflubenzuron in *Salmonidae*.

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<sup>1</sup> Commission Implementing Regulation (EU) No 2019/1881, O.J. L 290, of 11 November 2019

<sup>2</sup> [EFSA Journal 2012;10\(9\):2870. Conclusion on the peer review of the pesticide risk assessment of confirmatory data submitted for the active substance Diflubenzuron](#)

<sup>3</sup> Diflubenzuron assessment report, available at [http://dissemination.echa.europa.eu/Biocides/ActiveSubstances/0062-18/0062-18\\_Assessment\\_Report.pdf](http://dissemination.echa.europa.eu/Biocides/ActiveSubstances/0062-18/0062-18_Assessment_Report.pdf)

<sup>4</sup> [EFSA Journal 2015;13\(12\):4222. Peer review on the review of the approval of the active substance diflubenzuron regarding the metabolite PCA](#)



Subsequently the Commission recommended on 25 June 2019, the modification of the maximum residue limit in *Salmonidae*. This recommendation was confirmed on 16 July 2019 by the Standing Committee on Veterinary Medicinal Products and adopted by the European Commission on 8 November 2019.

# Summary of the scientific discussion for the establishment of MRLs

## 1. Introduction

Diflubenzuron, [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea] is an acyl urea derivative for use in the treatment of sea lice (*Lepeophtheirus salmonis*) infestations in Atlantic salmon. Diflubenzuron is administered as a 90% pre-concentrate in pelleted diet at a final concentration of 0.6 g diflubenzuron/kg with an intended oral dosage of 3-6 mg diflubenzuron/kg bw/day during 14 consecutive days.

The substance also has a history of use as a pesticide in agriculture and as a biocide, although these uses are now restricted. Diflubenzuron acts by interference with the synthesis of chitin. The demand for chitin is greatest at the moult between growth stages, and hence insects are killed due to disruption of the moulting process.

Diflubenzuron was previously assessed by the CVMP in 1998, and a toxicological ADI of 12.4 µg/kg bw, i.e 744/µg/person, was established based on the NO(A)EL of 1.24 mg/kg bw, derived from a long-term toxicity/carcinogenicity study in mice, which was considered as the most sensitive species, and applying a safety factor of 100.

Currently, diflubenzuron is included in Commission Regulation (EU) No 37/2010 of 22 December 2009 in accordance with the following table:

<b>Pharmaco- logically active substance</b>	<b>Marker residue</b>	<b>Animal species</b>	<b>MRLs</b>	<b>Target tissues</b>	<b>Other provisions</b>	<b>Therapeutic classification</b>
Diflubenzuron	Diflubenzuron	<i>Salmonidae</i>	1000 µg/kg	Muscle and skin in natural proportions	NO ENTRY	Antiparasitic agents / Agents against ectoparasites

On 7 May 2014 the European Commission submitted to the European Medicines Agency a request under Article 11 of Regulation (EU) No 470/2009 to issue a new opinion on diflubenzuron taking into account the genotoxic potential of diflubenzuron's metabolite 4-chloroaniline, and the more recent evaluations of diflubenzuron as a pesticide, undertaken by the European Food Safety Authority (EFSA)<sup>5</sup>, and as a biocide, coordinated by and the Commission's Joint Research Centre (JRC)<sup>6</sup>.

Subsequently, in May 2015, the CVMP adopted an opinion noting that the genotoxic metabolite has not been confirmed to be present in fish muscle and recommending that the previous MRL for diflubenzuron should be converted to a provisional MRL while data is sought on formation and depletion of the metabolite in fish muscle.

In March 2017, having considered the recommendation, the Commission clarified that Regulation (EC) No 470/2009 only allows the establishment of a provisional MRL in cases where scientific data are incomplete and where there are no grounds for supposing that residues of the substance at the level proposed constitute a hazard to human health. In the case of diflubenzuron there is a possibility that the genotoxic

<sup>5</sup> [EFSA Journal 2012;10\(9\):2870. Conclusion on the peer review of the pesticide risk assessment of confirmatory data submitted for the active substance Diflubenzuron](http://efsa.europa.eu/EFSA/Journal/2012/10/9/2870)

<sup>6</sup> Diflubenzuron assessment report, available at [http://dissemination.echa.europa.eu/Biocides/ActiveSubstances/0062-18/0062-18\\_Assessment\\_Report.pdf](http://dissemination.echa.europa.eu/Biocides/ActiveSubstances/0062-18/0062-18_Assessment_Report.pdf)

metabolite (4-chloroaniline) is present in treated fish at levels that could be hazardous to human health, and consequently the establishment of a provisional MRL was not considered appropriate. The Commission also highlighted the EFSA 2015 conclusion<sup>7</sup> relating to the use of diflubenzuron in plant protection products, indicating that the available data were not sufficient to demonstrate that the representative uses were safe for consumers. In light of the above the Commission invited the CVMP to revise its opinion of May 2015.

Before finalising its opinion, the CVMP invited interested companies to submit any data that might be helpful in further characterising the risk to the consumer associated with exposure to the metabolite 4-chloroaniline resulting from treatment of salmon with diflubenzuron. In response a newly performed residue study was provided, as reported in section 2.3.2 of this report.

During the current review both EFSA and ECHA were consulted, in accordance with Article 4(2) of Regulation (EC) No 470/2009 in connection with Recital 2 of the preamble to Regulation (EC) No 470/2009.

## 2. Scientific risk assessment

The consumer safety of diflubenzuron was assessed by the CVMP in 1998 for the purpose of establishing maximum residue limits in *Salmonidae* (see published CVMP summary report EMEA/MRL/621/99-Final). The information presented in that report is detailed below along with additional information taken from the pesticide and biocide evaluations, and relating to the genotoxic potential of diflubenzuron's metabolite, 4-chloroaniline, as well as additional residues data.

### 2.1. Safety assessment

#### 2.1.1. Overview of pharmacological properties

##### Pharmacodynamic properties including mode of action

No specific studies on pharmacodynamic properties of diflubenzuron were available.

##### Pharmacokinetic properties (mainly laboratory animals)

For the MRL evaluation carried out by the CVMP in 1998 pharmacokinetic studies in rats (single oral doses of 4 to 1000 mg/kg bw, repeated oral dose of 5 mg/kg bw/day for 14 days and single dermal doses of 0.05 and 0.5 mg/10 cm<sup>2</sup>), mice (single oral doses of 12.5 to 925 mg/kg bw), rabbits (single oral dose of 1 mg/kg bw and single dermal dose of 150 mg/kg bw) with unlabelled or radiolabelled diflubenzuron were available.

In rats diflubenzuron is absorbed from the gastrointestinal tract. Following a dose of 4 mg/kg bw 42.5% was absorbed, but following a dose of 900 mg/kg bw only 3.7% of the dose was absorbed. The absorption decreases with increasing dose indicating that the absorption is saturable in rats. In mice a similar pattern was observed. The maximum plasma concentration was 844 ng equivalents/ml in rats 4 hours after a single oral administration of 5 mg/kg bw. Diflubenzuron is rapidly and evenly distributed to all tissues. After administration of a single oral dose of 5 mg [<sup>14</sup>C]-diflubenzuron/kg bw to rats the highest mean levels of radioactivity at 4 hours were found in fat (4672 µg equivalents/kg), ovaries (3737 µg equivalents/kg), liver (2265 µg equivalents/kg), heart (1345 µg equivalents/kg), kidney (1200 µg

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<sup>7</sup> [EFSA Journal 2015;13\(12\):4222.Peer review on the review of the approval of the active substance diflubenzuron regarding the metabolite PCA](#)

equivalents/kg) and brain (984 µg/kg equivalents/kg). From 48 hours after administration onwards, the highest levels were retained in liver (mean level at 48 hours: 431 µg equivalents/kg) and erythrocytes (mean level at 48 hours: 379 µg equivalents/kg). No difference between males and females was noted. Absorption and distribution patterns were comparable after single and multiple administration and thus no accumulation of diflubenzuron is expected. Dermal absorption in rats and rabbits was minimal (less than 1%).

In rats and mice the major route of elimination is via faeces as intact diflubenzuron, via bile and urine after absorption. After single administration the excretion is almost complete after 24 to 48 hours. In rats at doses of 4 mg/kg bw up to 28% of the dose could be found in urine, approximately 30% in bile and 36% in faeces. Biliary and urinary elimination decreases with increasing dose in a dose dependent manner. Following repeated administration the excretion of diflubenzuron and metabolites was slightly slower than after a single dose in rats, being almost complete after 48 to 96 hours. In rabbits, urinary elimination seems to be the major route. After a single oral dose of 1 mg/kg bw 62% of the dose was excreted in urine after 48 hours. Less than 1% of an oral dose is recovered in exhaled air.

The major route of metabolism in rats is via hydroxylation of the phenyl moieties of diflubenzuron (approximately 80%). Another pathway is cleavage of the benzoyl-ureido bridge (20%). In urine and bile from rats the major metabolites were determined by HPLC or TLC/MS and identified as 2,6-difluoro-3-hydroxy-diflubenzuron, 2,6-difluorobenzoic acid, 2-hydroxy-diflubenzuron and 4-chloro-2-hydroxy- and 4-chloro-3-hydroxy-diflubenzuron. Other metabolites were 2,6-difluorohippuric acid and 2,6-difluorobenzamide. The cleavage product 4-chlorophenyl urea is also found but to a lesser extent (approximately 3 to 5%). In rats given a very high dose of diflubenzuron (100,000 mg/kg feed, equal to 7.8 g/kg bw/day) for 4 days, the metabolite 4-chloroaniline was detected in urine, although in very low concentrations (less than 0.01% of the dose absorbed).

Publicly available reports indicate that in sheep, swine and chicken, 4-chloroaniline has been found as a minor metabolite. In cattle, no or very low levels have been found<sup>8</sup> (EHC 184). The presence of 4-chloroaniline has been demonstrated in metabolism studies in apples and mushrooms.

Further to the Commission's request for review of its original opinion additional information available in the public domain (i.e. the data referred to in the assessments performed to the pesticides and biocides evaluations) were considered. In the evaluation of diflubenzuron for use as a pesticide it is stated that 4-chloroaniline as a metabolite in both humans and rats should be considered as a transient non-isolatable metabolite after exposure to diflubenzuron and the rat should be considered an appropriate model for human exposure to diflubenzuron. This statement is supported by data from an *in vitro* metabolism study using rat, pig, goat and human hepatocytes, which indicates that following incubation for 3 hours 4-chloroaniline concentrations are highest in the goat and pig and following incubation for 24 hours they are highest in the rat and pig. Concentrations of 4-chloroaniline were low in the human *in vitro* incubations at both time points analysed and no unique human metabolites were formed. While the relevance of these *in vitro* data to the *in vivo* situation may be questioned (because of uncertainty relating to, for example, influence of factors such as cell handling (collection, storage) and test media), it is considered that the data presented suggest that diflubenzuron is most readily metabolised by pigs, followed by rats, followed by goats, followed by humans, with less formation of 4-chloroaniline in humans than in rats.

From all information available it can be concluded that there are species differences in the metabolism of diflubenzuron. The genotoxic metabolite, 4-chloroaniline, is found in rats but only when given at very high doses. This substance is a metabolite in both humans and rats and the rat can be considered an appropriate model for human exposure to diflubenzuron. This is in line with the conclusions of the evaluation of diflubenzuron for use as a pesticide.

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<sup>8</sup> Environmental Health Criteria 184, (1996)

## 2.1.2. Calculation of a pharmacological ADI

A pharmacological ADI was not derived. This is acceptable as the mechanism of action of diflubenzuron is not considered relevant for mammals.

## 2.1.3. Overview of toxicology

### ***Single-dose toxicity***

Diflubenzuron was shown to have low acute toxicity in all tested species (rats, mice and rabbits). After oral administration of diflubenzuron in rats and mice the LD<sub>50</sub> was higher than 4640 mg/kg bw. After dermal exposure of diflubenzuron in rats and rabbits the LD<sub>50</sub> was higher than 2000 mg/kg bw. The intraperitoneal LD<sub>50</sub> in mice was higher than 2150 mg/kg bw. After inhalation of diflubenzuron the LC<sub>50</sub> in rats and rabbits was more than 2.49 mg/l air and 3.75 mg/l air, respectively. The only signs of toxicity seen were lethargy, slight dermal irritation and slight decreases in body weight gain in rabbits.

### ***Repeated dose toxicity***

Fourteen repeated dose toxicity studies were performed. Diflubenzuron was evaluated in four strains of rats (SPF, ALA:CFY rats, orally for 4 weeks, Wistar rats orally for 13 weeks, Sprague Dawley rats, orally for 13 weeks and 9 weeks, inhalation for 3 weeks, Charles River CrI:CD rats, dermal for 3 weeks), two strains of mice (CFLP mice, orally for 6 weeks and 14 weeks, B6C3F1 mice, orally for 13 weeks), dogs (Beagle, orally for 6 weeks, 13 weeks and 52 weeks) and rabbits (New Zealand White, dermal for 3 weeks and inhalation for 3 weeks). Oral doses of diflubenzuron were 3.125 to 100 000 mg/kg feed in rats, 16 to 50 000 mg/kg feed in mice and 10 to 160 mg/kg feed or 2 to 250 mg/kg bw in dogs. Dermal doses were 20 to 1000 mg/kg bw/day in rats and 69.6 to 322.5 mg/kg bw/day in rabbits. Doses of diflubenzuron after inhalation were 0.12 to 1.85 mg/l air in rats and 0.15 to 1.99 mg/l air in rabbits. In some of these studies the doses in mg/kg bw were not given.

The primary target organs for the toxicity of diflubenzuron after repeated administration were erythrocytes, the liver and the spleen. Diflubenzuron causes methaemoglobinaemia and sulfhaemoglobinaemia. Dose dependent methaemoglobinaemia was demonstrated after oral, dermal and inhalatory exposure. This effect is the most sensitive toxicological parameter. The 4-chloroaniline concentration in erythrocytes (up to 392 ng/g) may explain the occurrence of methaemoglobinaemia. Other toxicological effects reported were increased activity of liver enzymes, increased organ weights and histopathological findings, e.g. haemosiderosis and mild cell necrosis.

No observed effect levels (NOELs) could only be established in the oral 13-week study in Wistar rats as 12.5 mg/kg feed and in the 6-week oral study in mice as 16 mg/kg feed, equal to 2 mg/kg bw/day. However, a limited range of parameters was investigated in the mouse study. In the dog studies NOELs after oral administration could be established in the 6-week study as 4 mg/kg bw/day and in the 52-week study as 2 mg/kg bw/day. The lowest NOEL established in the dog studies was 2 mg/kg bw/day (52-week oral study) based on methaemoglobin formation.

### ***Target animal safety***

Tolerance studies have been performed in Atlantic salmon. Diflubenzuron was well tolerated in Atlantic salmon at single doses up to 1030 mg/kg bw (333 times the recommended dose) and after repeated doses up to 100 mg/kg bw (33 times the recommended dosage level) for 21 days (50% longer duration than the recommended). No significant adverse effects were observed on mortality, behaviour, inappetence or histopathological lesions.

### **Reproductive toxicity, including developmental toxicity**

Reproductive toxicity studies were performed in rats and rabbits.

Two 2-generation studies in rats (CD strain) with doses up to 160 mg/kg feed and doses up to 50 000 mg/kg feed, respectively, and a one-generation study in rats (ALA:CFY strain) with the doses 1000 and 100 000 mg/kg feed were conducted. There were no treatment-related effects on mating performance, pregnancy rate, duration of gestation, litter parameters or on the type and distribution of abnormalities in the studies. However, in the one-generation study and in the two-generation study with doses from 500 up to 50 000 mg/kg feed in rats dose related toxic effects were seen both in the parent generation and the offspring from all treatment groups. The toxic effects demonstrated were in the liver and spleen e.g. increased weights and centrilobular hepatocyte enlargement and on haematological parameters, e.g. increased values of methaemoglobin in the parents of both generations. A dose of 50 000 mg/kg feed induced adverse effects on the pre-weaning development of offspring. Thus, a NOEL for maternal toxicity could not be established from these studies but a NOEL for reproductive functions of 50 000 mg/kg feed (corresponding to 2403 to 8020 mg/kg bw) can be established.

Two teratogenicity studies have been performed in rats (strain CD) using oral doses of 1 to 4 mg/kg bw and 1000 mg/kg bw, respectively, and two studies in rabbits (New Zealand white) using oral doses of 1 to 4 mg/kg bw and 1000 mg/kg bw, respectively. None of the doses elicited maternal toxicity or any evidence of foetotoxicity or teratogenicity. For these effects the NOEL could be established as more than 1000 mg/kg bw.

### **Genotoxicity**

The mutagenic potential of diflubenzuron was investigated in a battery of properly conducted *in vitro* and *in vivo* studies. Tests for gene mutations in prokaryotic systems (five Salmonella-microsomal assays) and in eukaryotic systems (L5178Y mouse lymphoma cells, unscheduled DNA synthesis in rat hepatocytes and in W1-38 cells) as well as tests for chromosomal aberrations (mammalian cytogenetic test, micronucleus test, dominant lethal test in mouse) have been performed. All studies showed negative results for diflubenzuron. In addition, two *in vitro* studies (host-mediated transplacental carcinogen assay and malignant transformation in BALB/3T3 cells) were performed which showed that diflubenzuron did not induce cellular transformation. Thus, it can be concluded that diflubenzuron is devoid of mutagenic potential.

The genotoxicity of the metabolite 4-chloroaniline was not addressed in the CVMP summary report on diflubenzuron (CVMP summary report EMEA/MRL/621/99-Final). It is noted that the pesticides evaluation concludes that 4-chloroaniline is an *in vivo* genotoxic agent (see details in section 2.1.7).

### **Carcinogenicity**

Two long-term toxicity/carcinogenicity studies were performed in Sprague-Dawley rats where diflubenzuron was administered in the diet for 104 weeks. In the first study (non-GLP) dose levels of 0, 10, 20, 40 and 160 mg/kg feed, equal to 0, 0.35, 0.70, 1.43 and 5.83 mg/kg bw/day for males and 0, 0.43, 0.88, 1.73 and 7.05 mg/kg bw/day for females were used, and in the second study (GLP-compliant) dose levels of 0, 156, 625, 2500 and 10 000 mg/kg feed, equal to 0, 5.8 to 12.5, 23.7 to 49.5, 91.4 to 194.3 and 238 to 790 mg/kg bw/day for males and 0, 7.4 to 15.5, 28.2 to 63.9, 94.7 to 248 and 493 to 1156 mg/kg bw/day for females were used. The tumour profile of treated rats was similar to that of controls in both studies and there was no evidence to suggest that diflubenzuron is carcinogenic in rats. However, in the non-GLP study, survival was too low. Based on elevated levels of methaemoglobin, the NOEL in this study was 40 mg/kg feed, equal to 1.43 mg/kg bw/day for males and 1.73 mg/kg bw/day for females. In the GLP-study no NOEL could be retained as effects (decreased values for the myeloid :

erythroid ratio, elevated methaemoglobin and sulfhaemoglobin values and haemosiderosis) were seen in rats at the lowest dose tested (5.8 to 12.5 mg/kg bw).

In a 80-week non-GLP tumourigenicity study in CFLP mice, diflubenzuron was given via the diet at dose levels of 0, 4, 8, 16 and 50 mg/kg feed, corresponding to 0, 0.34, 0.67, 1.39 and 4.30 mg/kg bw/day for males and 0, 0.42, 0.80, 1.58 and 4.87 mg/kg bw/day for females. A positive trend in the incidence of lymphosarcoma was seen in female mice killed at termination. However, pair-wise comparisons resulted in significance only in the 8 mg/kg feed group and the combined findings of lymphosarcoma in the treated female mice dying during the treatment and killed at termination of the study was not significantly different from control. The incidence of all types of lymphoreticular tumours in female mice dying during the study or combined with those killed after 80 weeks was not statistically significant.

This was confirmed in another long-term toxicity/carcinogenicity study (GLP-compliant) in HC/CFLP mice at dosage levels of 0, 16, 80, 400, 2000 and 10 000 mg/kg feed, equal to 0, 1.24, 6.40, 32.16, 163.29 and 835.55 mg/kg bw/day for males and 0, 1.44, 7.26, 35.38, 186.59 and 958.51 for females, for 91 weeks. In this study the highest incidence of lymphosarcoma was seen in the control group of both males and females. The overall incidence and distribution of tumours found in this study was considered to be within the spontaneous tumour profile of the strain of mouse used. At doses at or above 400 mg/kg feed effects similar to those in the repeated dose toxicity studies were seen in the liver and spleen. The dosage level of 16 mg/kg feed, corresponding to 1.24 and 1.44 mg diflubenzuron/kg bw/day for males and females respectively, can be regarded as the NO(A)EL in this study despite occasionally statistically significant increases of relative methaemoglobin (% of total haemoglobin) values at week 52 for males and relative sulfhaemoglobin (% of total haemoglobin) levels at week 52 for both males and females and at week 78 for females. However, these effects were only transient and as the values in the control group also varied considerably, they are considered minor and, at this dosage, of no biological and toxicological significance.

The overall conclusion from the four rat and mouse studies, taking into consideration the negative results of the mutagenicity studies, is that there is no evidence for carcinogenicity of diflubenzuron in mice and rats.

The CVMP conclusion from the evaluation carried out in 1998 that there was no evidence of carcinogenicity of diflubenzuron is consistent with the more recent evaluations by Joint Research Centre<sup>3</sup> and EFSA<sup>2, 4</sup>. As reported in section 2.1.7, the rat can be considered an appropriate model for human exposure to parent compound, diflubenzuron.

The CVMP evaluation from 1998 did not address the metabolite 4-chloroaniline, but it is noted that the metabolite has been considered to be *in vivo* genotoxic by other scientific bodies.

#### **Studies of other effects including immunotoxicity**

Diflubenzuron (10% diluted in maize oil, 10% and 30% in vaseline and 1:1 mixture of Freund's adjuvant and maize oil) was tested for delayed hypersensitivity in albino guinea pigs. There was no statistical difference between treated and control animals with respect to the incidence of erythema and oedema. No evidence of delayed contact hypersensitivity in guinea pigs treated with diflubenzuron was seen.

The potential of diflubenzuron to produce skin or eye irritation was investigated in rabbits. Diflubenzuron was considered to be a marginal or very slight irritant to the eye. The skin irritancy of diflubenzuron technical grade could not be evaluated because of a poorly reported study. However, a formulation containing 92.2% diflubenzuron technical grade was considered to be non-irritating to rabbit skin.



#### **2.1.4. Calculation of the toxicological ADI**

In the evaluation carried out in 1998 for the establishment of maximum residue limits the CVMP established a toxicological ADI of 0.0124 mg/kg bw (equivalent to 744 µg/person) for diflubenzuron based on the NO(A)EL of 16 mg/kg feed, equal to 1.24 and 1.44 mg/kg bw/day for males and females, respectively, derived from the long-term toxicity/carcinogenicity study in the mouse, which was considered the most sensitive species, and applying a safety factor of 100.

#### **2.1.5. Overview of microbiological properties of residues**

Studies on microbiological properties of diflubenzuron were not available and are not considered to be necessary as the substance is not expected to exert antimicrobial activity.

#### **2.1.6. Observations in humans**

No studies were available on observations in humans.

#### **2.1.7. Findings of EU or international scientific bodies**

Diflubenzuron has been assessed by several international bodies. The main conclusions of their assessments are reported in the paragraphs below.

##### **Biocide evaluation coordinated by the European Commission's Joint Research Centre (JRC)**

In 2012 diflubenzuron was evaluated in the review-programme for biocides coordinated by JRC for use as an insecticide with regard to the control of termites in house grounds, mosquito larvae in water systems and fly larvae<sup>3</sup>.

It was considered that in relation to these uses where the products are not expected to contaminate food, feeding stuffs or livestock the establishment of an ADI was not relevant. However, the biocide evaluation concluded that for the establishment of an ADI the NOAEL from the long term toxicity/carcinogenicity (91-week) study in mice would be the appropriate point of departure from which to establish the ADI, applying a safety factor of 100. This conclusion is consistent with the conclusion of the CVMP evaluation carried out in 1998.

##### **European Food Safety Authority (EFSA)**

EFSA evaluated diflubenzuron for its use in plant protection products<sup>2, 4</sup>. Based on the data available EFSA concluded that the weight of evidence suggests that 4-chloroaniline is an *in vivo* genotoxic agent.

The evaluation by EFSA considers that potential exposure to 4-chloroaniline can occur as a metabolite via intake of, or exposure to, diflubenzuron or as an impurity present in the diflubenzuron batches or as a residue (i.e. direct exposure to 4-chloroaniline). The experts at the Pesticides Peer Review Meeting on mammalian toxicology in 2012 concluded that 4-chloroaniline as a metabolite in both humans and rats should be considered as a transient non-isolatable metabolite after exposure to diflubenzuron.

The rat was considered an appropriate model for human exposure to diflubenzuron and, in the rat, genotoxic and carcinogenic potential of diflubenzuron were not observed.

EFSA established an ADI of 0.1 mg/kg bw/day for diflubenzuron based on a 1-year dog study.

4-Chloroaniline was further considered in the 2015 EFSA report, where benchmark dose lower limits (BMDL) were derived from a rat carcinogenicity study undertaken by the US National Toxicology Program

(NTP)<sup>9</sup>. In this study the doses 0, 2, 6 and 18 mg/kg were administered by gavage. In male rats the incidences of pheochromocytomas of the adrenal glands was 13/49, 14/48, 14/48 and 25/49 respectively. A BMDL10 (the dose estimated to cause a 10% incidence of tumours) and a BMDL5 (the dose estimated to cause a 5% incidence of tumours) of 0.56 and 0.16 mg/kg bw, respectively, were derived by EFSA.

### **Joint FAO/WHO Meeting on Pesticides Residues (JMPR)**

The Joint FAO/WHO Meeting on Pesticides Residues (JMPR) evaluated diflubenzuron in 1981, 1984 and 1985 and established an ADI of 0.02 mg/kg bw based on NOELs for methaemoglobin formation in the submitted long-term toxicity/carcinogenicity studies in dogs (2 mg/kg bw), rats (40 mg/kg feed, corresponding to approximately 2 mg/kg bw) and mice (16 mg/kg feed, corresponding to approximately 2.4 mg/kg bw) and applying a safety factor of 100. The values for feed consumption were standard figures and not those measured in the rat and mouse studies.

### **Joint FAO/WHO Expert Committee on Food Additives (JECFA)**

The JECFA evaluated diflubenzuron for use in fish in 2015. In the absence of adequate information on exposure to 4-chloroaniline, and on whether diflubenzuron can be metabolized to 4-chloroaniline in humans, the Committee was unable to establish an ADI for diflubenzuron because it was not possible to assure itself that there would be an adequate margin of safety from its use as a veterinary drug.

### **International Programme on Chemical Safety (IPCS).**

Diflubenzuron was evaluated by the International Programme on Chemical Safety in 1996 and an ADI established of 0.02 mg/kg bw based on NOELs for methaemoglobin formation in the submitted long-term toxicity/carcinogenicity studies in dogs (2 mg/kg bw), rats (40 mg/kg feed, corresponding to approximately 2 mg/kg bw) and mice (16 mg/kg feed, corresponding to approximately 2.4 mg/kg bw) applying a safety factor of 100.

4-Chloroaniline was evaluated in 2003. The evaluation concluded that the substance is rapidly absorbed and metabolised. Reactive metabolites bind covalently to haemoglobin and to proteins in the liver and kidneys. Results from an NTP study<sup>5</sup> showed that 4-chloroaniline was carcinogenic in male rats with the induction of unusual and rare tumours of the spleen (fibrosarcoma and osteosarcoma). In the female rats, the pre-cancerous stages of the spleen tumours were increased in frequency. Increased incidences of pheochromocytoma of the adrenal gland in rats were related to 4-chloroaniline administration. An NTP carcinogenicity study was also performed in mice<sup>5</sup>, where there was some evidence of carcinogenicity in males mice, indicated by hepatocellular tumours and haemangiosarcoma.

4-Chloroaniline showed transforming activity in cell transformation assays. A variety of *in vitro* genotoxicity tests (e.g. Salmonella mutagenicity test, mouse lymphoma assay, chromosomal aberration test, induction of sister chromatid exchange) indicated that 4-chloroaniline is possibly genotoxic, although results were sometimes conflicting. Due to lack of data, it was impossible to reach any conclusion on the *in vivo* genotoxicity of 4-chloroaniline.

A calculated tolerable daily intake of 2 µg/kg bw was set, however this was based only on non-neoplastic effects. It was also stated that residual levels of 4-chloroaniline in consumer products should be further reduced or entirely eliminated.

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<sup>9</sup> NTP (1989) Toxicology and carcinogenesis studies of para-chloro-aniline hydrochloride (CAS No. 20265-96-7) in F344/N rats and B6C3F1 mice (gavage studies). NIH Publication No. 89-2806

### 2.1.8. Overall conclusions on the ADI

In its 1998 evaluation the CVMP did not set a pharmacological or microbiological ADI. The toxicological ADI of 0.0124 mg/kg bw (equivalent to 744 µg/person) was established as the overall ADI.

The ADI is set for diflubenzuron, but the genotoxic and carcinogenic metabolite 4-chloroaniline is a substance of concern and the potential risk for consumers needs to be addressed. Genotoxic substances should not be used in veterinary medicinal products for food producing animals because, in principle, there is no threshold below which it is possible to conclude that exposure will not result in cancer causing mutations. However, at low doses DNA-repair and cell proliferation are important modifying factors and the contribution of very low doses of substances that are both genotoxic and carcinogenic to background DNA damage (which even in normal physiology is considerable) may be negligible. Therefore, the notion that there is no threshold below which cancer causing mutations will occur may overestimate the real risk.

Consequently, potential exposure to a genotoxic carcinogen, at very low levels may be acceptable as long as it is clear that the quantity to which a consumer could potentially be exposed is low enough to be considered virtually safe. In practice this means that the level shall be low enough to ensure that the increased cancer risk that would result from consumer exposure to the substance would be less than 1 in 10<sup>6</sup>.

An exposure limit associated with this level of risk may be determined either using chemical specific data or, in the absence of such data, using the threshold of toxicological concern (TTC) concept.

Substance specific carcinogenicity data exist for 4-chloroaniline. These data can be used to calculate the daily dose associated with a cancer risk of 1 in 10<sup>6</sup>. One possibility is to use the BMDL values derived by EFSA as starting points. The BMDL5 (0.16 mg/kg) and BMDL10 (0.56 mg/kg), provide estimates of the lowest dose which is 95% certain to cause no more than a 5% and 10% cancer incidence, respectively. To calculate the dose associated with a cancer incidence of 1 in 10<sup>6</sup> the BMDL5 and BMDL10 need to be divided by 50 000 and 100 000, respectively, resulting in figures of 0.2 µg/person ([0.16 mg/kg\*60 kg]/50,000) and 0.35 µg/person ([0.56 mg/kg\*60 kg]/100,000), depending on whether the BMDL5 or BMDL10 is used as starting point.

An alternative approach used to calculate the dose associated with a particular increased cancer incidence uses the following equation:

$$D_h^x = \frac{I_{human} \times t_{exp} \times t_{exp\ osure}}{I_{exp} \times t_{life} \times t_{life}} \times d_{exp}$$

$D_h^x$  : dose for humans at accepted cancer risk;

$I_{human}$  : accepted lifetime cancer risk (1 in 10<sup>6</sup>);

$I_{exp}$  : tumour incidence at lowest tumorigenic dose animal experiment minus tumour incidence in control (0.29 - 0.26);

$t_{exp}$  : duration of animal experiment in days (721 (103 weeks));

$t_{life}$  : duration of lifetime of experimental animals in days (rat 1000)

$t_{exposure}$  : duration of exposure in days (721);

$d_{exp}$  : lowest tumorigenic dose (2 mg/kg).

Based on this formula and using the incidence of rat adrenal gland pheochromocytomas as a starting point, the dose of 4-chloroaniline calculated to result in an excess cancer risk of 1 in 10<sup>6</sup> is 2 µg/person. While this approach has not been used by CVMP previously, it has been used for risk assessments by

some national competent authorities<sup>10</sup>. This method has the benefit of providing a value associated with an increased cancer risk of 1 in 10<sup>6</sup> rather than simply an overall cancer risk of 1 in 10<sup>6</sup> (including background incidence).

Finally, the threshold of toxicological concern (TTC) approach provides human exposure threshold values below which there is a very low probability of adverse effects to human health (European Union, 2012 SCCS/SCHER/SCENIHR). The approach is applicable to substances for which the chemical structure is known but for which there are few or no relevant toxicity data. For substances with a structural alert for genotoxicity a TTC of 0.15 µg/person/day is generally accepted as the threshold below which human exposure should remain (and is associated with an increased cancer risk of 1 in 10<sup>6</sup>).

In summary, for chloroaniline, a number of different approaches can be used to establish the dose associated with an acceptably low cancer risk.

## **2.2. Residues assessment**

### **2.2.1. Pharmacokinetics in target species**

The pharmacokinetics of diflubenzuron in the target species Atlantic salmon was studied after a single dose of 75 mg/kg of radioactively (<sup>14</sup>C) labelled diflubenzuron at +8°C and after a single intravenous dose and oral dose of 3 mg/kg bw at a water temperature of +6°C.

Diflubenzuron was only partially absorbed from the gastrointestinal tract. After administration of high doses (75 mg/kg, i.e. 12.5 to 25 times the recommended dose) only 3.7% of the dose was absorbed after 12 hours. After administration of the recommended dose (3 mg/kg), the bioavailability was calculated to be 31% at a water temperature of +6°C. The absorption of diflubenzuron is therefore considered to be dose dependent and saturable in the target species. The kinetics of diflubenzuron after oral treatment at a water temperature of +6°C followed a one-compartment open model with first order input and first order output with a lag time of 3.5 hours. The mean peak plasma level (0.141 µg/ml) was reached after 24 hours. Autoradiography showed distribution of diflubenzuron residues in the liver, kidney, brain, bile, fat and cartilage. The highest recovery, 10% of the administered dose, was found in the fillet (muscle and skin in natural proportion) 1 day after administration. The highest concentrations were found in the liver although they only accounted for less than 0.3% of the given dose. The radioactivity in bile was very high indicating that the biliary route is the major excretory pathway. The elimination half-life at +6°C was calculated to be 71.4 hours.

The metabolism in salmon was studied after single (single dose of radiolabelled diflubenzuron) or multiple administration (13 days of feeding of unlabelled diflubenzuron followed by a single dose of radiolabelled diflubenzuron) at the recommended dose of 3 mg/kg bw (water temperature +15°C). Diflubenzuron was metabolised and rapidly excreted, mainly via the bile. Six hours after administration 39% of the radioactivity in bile was identified as diflubenzuron. One and 4 days after administration most of the radioactivity in bile derived from water-soluble metabolites. Chromatographic analysis with radio-HPLC of fillet revealed three components. The major component was identified as diflubenzuron, 98.75%, 99.16% and 99.47% of total residues as determined by chromatography after 1, 4 and 7 days, respectively after repeated administration (13 days feeding of unlabelled diflubenzuron and 1 day with radiolabelled compound given 3 mg/kg orally) and 97.39% of total residues as determined by chromatography 1 day after administration of a single dose of radiolabelled diflubenzuron. Furthermore, one metabolite was identified as 4-chlorophenyl urea with maximum concentration of 0.23 µg/kg at 4 days after

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<sup>10</sup>Linders JBHJ (1990) Risicobeoordeling voor de mens bij blootstelling aan stoffen. Uitgangspunten en veronderstellingen [Risk assessment to humans after exposure to substances. Starting points and assumptions] RIVM report no. 725201003.

administration. The third component was not identified (less than 7 µg/kg) but the retention time was in the same range as for 4-chloroaniline. In the liver five components were found. Three components were identified as diflubenzuron, 4-chloroaniline (less than 3 µg/kg) and 4-chlorophenyl urea (less than 9 µg/kg). The two unidentified metabolites were probably mono-hydroxylated products of diflubenzuron. The finding in the metabolism study reported in the CVMP MRL summary report is not consistent with the evaluation of diflubenzuron by the IPCS where it is stated that 4-chloroaniline was not detected in fish.

### 2.2.2. Residue depletion studies

The concentration of marker residue (diflubenzuron) to total residues in salmon (weight 391 to 870 g) was evaluated after single and multiple administration (13 days feeding of unlabelled diflubenzuron and 1 day with radiolabelled compound) with the recommended dose of 3 mg/kg via gavage at a water temperature of +15°C. In fillet (muscle and skin) the total residues after repeated administration were 466, 117 and 26 µg equivalents diflubenzuron/kg at 1, 4 and 7 days after administration, respectively, and after single administration the values were 447 and 21 µg equivalents diflubenzuron/kg at 1 and 7 days, respectively. The total residues in liver after repeated administration were 811, 334 and 181 µg equivalents diflubenzuron/kg at 1, 4 and 7 days after administration, respectively, and after single administration the values were 943 and 192 µg equivalents diflubenzuron/kg at 1 and 7 days after administration, respectively.

Concentrations of diflubenzuron in fillet, analysed with radio-HPLC, after repeated administration (13 days feeding of unlabelled diflubenzuron and 1 day with radiolabelled compound given 3 mg/kg orally) were 389, 99.6 and 21.4 µg diflubenzuron/kg at 1, 4 and 7 days after administration, respectively, and after single dosing the value was 410 µg diflubenzuron/kg 1 day after administration. These values result in a ratio of marker residue versus total radioactive residues of 83%, 85% and 82% at 1, 4 and 7 days after administration in fillet (muscle and skin in natural proportions) after repeated administration and 92% at 1 day after administration of a single dose, reflecting the low metabolism in Atlantic salmon.

Three non-radiometric residue depletion studies were conducted in Atlantic salmon at water temperatures of +15°C and +6°C. The concentrations were measured with an HPLC method with UV detection and a quantification limit for diflubenzuron of 50 µg/kg.

Atlantic salmon (600 to 1346 g) were fed diflubenzuron daily as medicated feed *ad libitum* for 30 minutes each day, for 14 days at a water temperature of +15 °C and at a dose of 3.19 mg/kg bw. Liver and fillet were analysed on day 1, 7, 14 and 21 after treatment. In the fillet, 1550 (350 to 3080) µg/kg, 200 (70 to 330) µg/kg, less than 50 µg/kg and less than 50 µg/kg of diflubenzuron were measured (mean of 10 fish) on day 1, 7, 14 and 21 after treatment, respectively. In the liver, 2170 (720 to 3400) µg/kg, 260 (120 to 350) µg/kg, 40 (less than 50 to 80) µg/kg and less than 50 (less than 50 to 60) µg/kg of diflubenzuron were measured on days 1, 7, 14 and 21 after treatment, respectively. The individual values for each fish are in a broad range probably because of the different weights of the individual fish and of the feeding *ad libitum* which results in different doses in each individual fish.

In another study Atlantic salmon (619 to 1344 g) were fed diflubenzuron daily as medicated feed *ad libitum* for 30 minutes each day, for 14 days at a water temperature of +6°C or 1°C and at a dose of 2.9 mg/kg bw. Liver and fillet were analysed on days 1, 7, 14 and 21 after treatment. In the fillet, 2240 (980 to 3670) µg/kg, 400 (120 to 680) µg/kg, 100 (30 to 270) µg/kg and 40 (30 to 80) µg/kg of diflubenzuron were measured (mean of 10 fish) on day 1, 7, 14 and 21 after treatment, respectively. In the liver, 3190 (1790 to 4860) µg/kg, 730 (530 to 990) µg/kg, 120 (60 to 280) µg/kg and less than 50 µg/kg of diflubenzuron were measured on day 1, 7, 14 and 21 after treatment, respectively. The individual values for each fish are in a broad range probably because of the different weights of the individual fish and of the feeding *ad libitum* which results in different doses in individual fish.

One more study was performed in Atlantic salmon (5000 g) given approximately 2.66 mg diflubenzuron/kg bw as medicated pellets *ad libitum* 6 hours a day, at a water temperature of +14.6 °C to +15.5 °C for 14 days. Liver, muscle and skin samples were collected and analysed on day 5, 14, 21 and 28 after treatment. In the muscle, 900 (530 to 1900) µg/kg, 100 (less than 50 to 170) µg/kg, less than 50 (less than 50 to 500) µg/kg and less than 50 µg/kg of diflubenzuron were measured on day 5, 14, 21 and 28 after treatment, respectively. In the skin, 320 (less than 50 to 520) µg/kg of diflubenzuron was measured day 5 after treatment and less than 50 µg/kg the other days. In liver, 520 (less than 50 to 890) µg/kg, 70 (less than 50 to 150) µg/kg, less than 50 µg/kg and less than 50 µg/kg of diflubenzuron were measured day 5, 14, 21 and 28 after treatment. The individual values for each fish are in a broad range probably because of the feeding *ad libitum*, which results in different doses in each individual fish.

In these studies the marker residue, diflubenzuron, was analysed and there is no information on the metabolites. The results showed great variability, probably because of the different weights of the individual fish and the uncertainty in dosing with *ad libitum* feeding. The amount of residues was higher in colder water. However, all values were below the MRL 7 days after treatment.

A non-GLP-compliant field study was performed in 2017 to monitor 4-chloroaniline in Atlantic salmon during the withdrawal period of 105 degree days following feeding of diflubenzuron at a commercial farm. The fish (mean weight of 2921 g at start of treatment) were held in sea water with an average temperature of 9°C and fed a commercial medicated feed containing 0.6 g diflubenzuron/kg feed delivered using a commercial feeding system for 16 days with an achieved dose ranging from 0.6-3.8 mg/kg bw per day (the prescribed dose is 3-6 mg/kg for 14 days). Sampling of muscle and skin in natural proportions (cross section from the back of the dorsal fin to the anal vent) was performed pre-treatment, at end of treatment and 1 (14 and 24 hours), 3, 5, 8 and 12 days post treatment (n=12 per group, mean weight of fish was 3603 g). The vertebral section was removed and the skin plus muscle and fat was homogenized and analysed for content of diflubenzuron and 4-chloroaniline by using UHPLC/HRMS and LS-MS/MS, respectively. For 4-chloroaniline the LOD was 0.33 µg/kg and the LOQ was 1 µg/kg. For diflubenzuron the LOQ was 0.01 mg/kg.

The concentration of diflubenzuron was below the LOQ (0.01 mg/kg) in all pre-treatment samples. At day 8, or 77 degree days, all concentrations were below the MRL of 1000 µg/kg.

Quantifiable concentrations of 4-chloroaniline were measured in 1 sample at 14 hours after administration (1.27 µg/kg) and in 1 sample at 24 hours after administration (1.01 µg/kg). All other samples were below the LOQ. From the end of administration up to 3 days following treatment at least 50% of the samples were above the LOD. Five days after administration 1 sample was above the LOD and from 8 days after administration all samples were below the LOD.

### ***Selection of marker residue ratio of marker to total residues***

The CVMP previously concluded from the available studies that the parent compound diflubenzuron could be considered as the marker residue and the ratio of marker residue to total residues of 92% was calculated at 1 day after single administration.

### **2.2.3. Monitoring or exposure data**

Diflubenzuron monitoring data were made available by the Norwegian Food Safety Authority covering the period from the beginning of 2010 to November 2017. Out of 641 fish samples analysed 9 showed diflubenzuron at levels greater than the LOQ (which varied from 1 to 10 µg/kg), with a highest recorded residue level of 14 µg/kg. There are no monitoring data for 4-chloroaniline available.

## 2.2.4. Analytical method for monitoring of residues

The 1998 summary report on diflubenzuron includes reference to a validated analytical method for the determination of diflubenzuron residues in muscle and skin of salmon. However, the limit of quantification of that method was 50 µg/kg, which although adequate for the MRL recommended at that time (1000 µg/kg), is not sufficiently sensitive to allow determination of residues in the region of the revised MRL recommended now.

However, an analytical method used in residue control was provided by the Norwegian Food Safety Authority. The method uses LCMS/MS with a range of 1-1500 µg/kg. The method is generally well described and covers the range needed for the proposed MRL. The method was validated in accordance with Community reference laboratories residues (CRLs) 20/1/2010, Guidelines for the validation of screening methods for residues of veterinary medicines. Furthermore, the relevant laboratory is in the process of undertaking further validation in line with requirements of Commission Decision 2002/657/EC.

The relevant European Reference laboratory has reviewed the proposed analytical method.

## 2.2.5. Findings of EU or international scientific bodies

Following the review of diflubenzuron in relation to its use as a biocide, the substance was included in Annex I to Directive 98/8/EC repealed by Regulation (EU) No 528/2012 with the following specific provision:

“For products containing diflubenzuron that may lead to residues in food or feed, Member States shall verify the need to set new or to amend existing maximum residue levels (MRLs) in accordance with Regulation (EC) No 470/2009 or Regulation (EC) No 396/2005, with special consideration to the *in vivo* genotoxic metabolite 4-chloroaniline, and take any appropriate risk mitigation measures ensuring that the applicable MRLs are not exceeded”.

The EFSA 2012 review of diflubenzuron<sup>2</sup> in relation to its use as a pesticide highlighted that potential exposure to 4-chloroaniline as a residue (i.e. either for consumers or for workers and bystanders/residents) should be considered a priori as a concern since a threshold for a genotoxic carcinogen cannot be assumed (i.e. an AOEL, ADI and ARfD cannot be set).

In 2015 EFSA published its opinion<sup>4</sup> on the potential exposure to 4-chloroaniline (as an impurity and metabolite of diflubenzuron) as a residue and its potential toxicological relevance. Since a threshold cannot be assumed for a genotoxic substance the potential exposure to 4-chloroaniline was considered to represent a concern. The level of risk associated with known uses of 4-chloroaniline was estimated using the margin of exposure (MoE) approach based on a point of departure established from animal carcinogenicity studies where the BMDL10 and BMDL5 were 0.56 and 0.16 mg/kg bw respectively. It was also noted that a significant transformation (57%) of diflubenzuron residues into 4-chloroaniline was observed in an experiment under conditions simulating the sterilization of food at a temperature of 120 °C. However, no transformation of diflubenzuron to 4-chloroaniline was seen at temperatures at or below 100°C. The MOE was calculated and was lower than the minimum recommended value of 10 000 for several food items. In the final review report from the European Commission in 2017<sup>11</sup> it was concluded that the use of diflubenzuron should be restricted to uses for which an exposure of consumers to 4-chloroaniline can be excluded. Commission Implementing Regulation 2017/855 of 18 May 2017<sup>12</sup> indicates that (in relation to use in plant protection products) diflubenzuron is restricted to uses as an

<sup>11</sup> [Commission staff working document of 23 March 2017](#), Final Review report for the active substance diflubenzuron, Diflubenzuron SANCO/831/08 – rev 6 23 March 2017

<sup>12</sup> [Commission Implementing Regulation 2017/855](#) of 18 May 2017 amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance diflubenzuron

insecticide in non-edible crops.

Codex Alimentarius has established MRLs for diflubenzuron in various vegetable and animal commodities with regard to its use as pesticides. However, no MRLs in fish have been established.

### **3. Risk management considerations**

#### **3.1. Potential effects on the microorganisms used for industrial food processing**

Diflubenzuron substance is not intended for use in dairy cattle and therefore potential effects in dairy products were not investigated.

#### **3.2. Other relevant risk management considerations for the establishment of maximum residue limits**

Sea lice represent a significant health and welfare problem for farmed salmon. Although a number of substances are available for the treatment of sea lice, resistance is reported in many of these (emamectin benzoate, hydrogen peroxide, azamethiphos and pyrethroids). No resistance is yet reported to flubenzurons and there is clear value in maintaining a range of treatment options in order to allow rotation of treatments and so further delay development of resistance.

Under commercial salmon farming conditions, salmon are starved for a period prior to, and during, transport to the place of slaughter. Typically, this period is in the region of 7 days. Therefore, it is considered highly unlikely that salmon will be presented for slaughter less than one week following the cessation of diflubenzuron treatment. This argument is supported by the Norwegian residue surveillance data where very few samples were positive for diflubenzuron residues and, of those that were positive, diflubenzuron residues were detected at concentrations far below the established MRL of 1000 µg/kg.

In addition, based on current fish farming practice and a knowledge of diflubenzuron use, it is considered unlikely that fish will be treated with diflubenzuron up to the point of slaughter. Indeed, recent usage data for current authorised products containing diflubenzuron indicate that diflubenzuron was used to treat 1.65% of farmed salmon in Norway in 2017. These data suggest that any potential for consumer exposure to diflubenzuron or the metabolite 4-chloroaniline will be infrequent. Again, this conclusion is supported by the 2010-2017 residue monitoring data from Norway.

#### **3.3. Elaboration of MRLs**

Diflubenzuron was originally assessed by the CVMP in 1998, which led to the establishment of the current MRL in *salmonidae* (1000 µg/kg in muscle and skin in natural proportions).

Based on this MRL value, the daily intake of diflubenzuron residues from salmon represents approximately 43% of the ADI. This is in line with the general approach taken by CVMP that, for substances used in both plant protection products as well as in veterinary medicinal products, not more than 45% of the ADI should be used up as a result of exposure via veterinary medicines.

However, this review of the MRL opinion for diflubenzuron focuses particularly on the genotoxic metabolite 4-chloroaniline and on whether use of diflubenzuron in veterinary medicines represents a hazard to consumers resulting from exposure to 4-chloroaniline.



## Estimation of consumer exposure to 4-chloroaniline

Consumer exposure to 4-chloroaniline may theoretically occur via (i) direct intake of 4-chloroaniline as a residue in food, (ii) via intake of diflubenzuron residues with subsequent metabolism to 4-chloroaniline and (iii) via transformation of diflubenzuron residues to 4-chloroaniline during food processing or cooking and ingestion of the resulting 4-chloroaniline.

In general, the intake calculation assumes that the marker residue will be present at the MRL level, which may not occur until some time after the end of treatment. However, as 4-chloroaniline is a genotoxic substance a more conservative approach is considered appropriate: residues of the metabolite in fish should remain at acceptable levels at all times following treatment, i.e. allowing for the possibility that treated animals may be slaughtered before the authorised withdrawal period elapses.

The worst case consumer exposure resulting from direct intake of 4-chloroaniline residues has been calculated using data from the residue study measuring the levels of 4-chloroaniline in fish muscle (salmon) after treatment with diflubenzuron (reported in section 2.2.2) and assuming ingestion of treated fish at time points before parent diflubenzuron depletes to the MRL. In two out of 24 samples taken on day 1 after treatment 4-chloroaniline was found above the LOQ of 1 µg/kg (one positive sample at 14 hours (1.27 µg/kg) and one at 24 hours (1.01 µg/kg)). The 4-chloroaniline levels in these two samples would result in a consumer exposure of 0.3 and 0.38 µg/day (1 and 1.27 µg/kg x 0.3 kg according to the food basket). However, the doses achieved in the residue depletion study were below, or in the low range, of the recommended dose (achieved doses of 0.6-3.8 mg/kg bw versus recommended doses of 3-6 mg/kg bw) and higher residue levels can be expected after higher doses. Worst case consumer exposure to 4-chloroaniline via fish treated with the maximum recommended dose of 6 mg diflubenzuron/kg was therefore estimated by doubling the maximum recorded residue level seen in the study, which results in an exposure of 0.76 µg 4-chloroaniline/person. It should be stressed that the above calculation is highly conservative as it is based on the highest 4-chloroaniline residues detected in two positive samples out of a total 24 samples harvested within 24 hours after the end of treatment.

Residue levels between the LOD (0.33 µg/kg) and LOQ (1 µg/kg) were seen up until day 5 after the end of treatment. If it is assumed that diflubenzuron concentrations in these samples were at the LOQ, then the worst case consumer exposure (based on 300 g fish intake and correcting (doubling) to take account of the sub-maximal dosing) 5 days after treatment would be 0.6 µg/person (1 µg/kg x 2 x 0.300 kg).

Indirect exposure to 4-chloroaniline as a result of ingestion of parent diflubenzuron in salmon tissues and subsequent metabolism in the consumer is not considered an exposure route of concern as the rat is considered an appropriate model for human exposure to diflubenzuron and data generated in the rat do not indicate that diflubenzuron has genotoxic/carcinogenic potential. That said, exposure to diflubenzuron in food and subsequent metabolism to 4-chloroaniline may add to exposure from other routes and contribute to the overall consumer exposure to 4-chloroaniline. Based on available data it can be estimated that up to 5% of parent diflubenzuron might be metabolised to 4-chloroaniline in the consumer. Assuming that the consumer is exposed to diflubenzuron at the established MRL of 1000 µg/kg salmon, the potential human exposure to 4-chloroaniline can be estimated to be 15 µg/person (MRL (1000 µg/kg) x daily consumption (0.300 kg) x conversion ratio (0.05)). However, as noted above, Norwegian monitoring data demonstrate that, in practice, few salmon samples are positive for diflubenzuron under conditions of field use and, when present, diflubenzuron is detected at concentrations well below the current MRL (the maximum residue detected in fish sampled in the field was 14 µg/kg).

Transformation of diflubenzuron to 4-chloroaniline during processing or cooking of salmon is likely to make only a relatively minor contribution to consumer exposure to the genotoxic substance. EFSA

investigated the production of 4-chloroaniline under conditions designed to mimic pasteurization (90°C in a climate chamber at a pH of 4.0 for 20 minutes), baking or boiling (100 °C in a climate chamber at a pH of 6.0 for 60 minutes) and sterilisation (120 °C in an autoclave at a pH of 6.0 for 20 minutes). 4-Chloroaniline was only detected under the most extreme conditions (sterilization). While these conditions may be relevant for canning of fish, the quantity of salmon produced in Europe that is processed by canning is expected to be very limited: the majority of salmon consumed in Europe is fresh or smoked, with smoked salmon accounting for 94% of all processed salmon<sup>13</sup>. Therefore, the results obtained under sterilization conditions are considered to be of limited relevance for the European situation. The data indicate that conversion to 4-chloroaniline occurs at temperatures somewhere between 100 and 120 °C, while the ideal temperature of cooked salmon meat is approximately 55-65°C. The temperature of the major part of the fillet can be expected to remain well below 100°C. However, the possibility that some parts of the fish may reach a higher temperature cannot be excluded. While an accurate estimation of the exposure to 4-chloroaniline that occurs as a result of cooking diflubenzuron-containing salmon is not feasible without appropriate data, a worst case estimate can be undertaken assuming that up to 5% of a fish fillet will be exposed to temperatures greater than 100°C (the data indicate that, at temperatures above 100°C 57% of diflubenzuron was converted to 4-chloroaniline). If it is further assumed that the fish fillet contains diflubenzuron at the current MRL of 1000 µg/kg, and a conversion of 57% of diflubenzuron to 4-chloroaniline occurs in 5% of the filet, then the potential worst case human exposure via this route is estimated to be 8.55 µg/person (MRL (1000 µg/kg) x daily consumption (0.300 kg) x % of fish fillet exposed to temperatures above 100°C (0.05) x conversion ratio (0.57)). However, again, based on the Norwegian monitoring data few salmon samples are positive for diflubenzuron under conditions of field use and, when diflubenzuron was present, it was detected at concentrations well below the current MRL (the maximum residue detected in fish sampled in the field was 14 µg/kg).

In summary, consumer exposure to 4-chloroaniline could, in principle, arise from direct intake of 4-chloroaniline as a residue in food; via intake of diflubenzuron residues with subsequent metabolism to 4-chloroaniline; and via transformation of diflubenzuron residues to 4-chloroaniline during the cooking process and ingestion of the resulting 4-chloroaniline.

- A worst case consumer exposure estimate as a result of direct exposure to residues of 4-chloroaniline indicates that consumers could ingest up to 0.76 µg 4-chloroaniline/person if salmon were ingested within a day of treatment cessation.
- In practice, indirect exposure to 4-chloroaniline resulting from metabolism of ingested diflubenzuron is not considered to represent a concern as the rat is considered an appropriate model for this type of exposure, and data generated in rats does not indicate that ingested diflubenzuron is associated with genotoxic or carcinogenic effects. However, a theoretical worst case exposure calculation performed assuming consumer exposure to diflubenzuron at the established MRL and metabolism of 5% of the diflubenzuron to 4-chloroaniline would result in an estimated exposure level of 15 µg 4-chloroaniline per person.
- Finally, in practice exposure to 4-chloroaniline as a result of transformation of diflubenzuron to 4-chloroaniline during cooking is likely to make only a minor contribution to the overall exposure to 4-chloroaniline, as the available data only demonstrate that this conversion occurs at temperatures in excess of those used during normal cooking. However, a theoretical worst case exposure calculation performed based on the assumption that 5% of a fish fillet will be exposed to temperatures at which conversion occurs, that where conversion occurs 57% of diflubenzuron will be converted into 4-chloroaniline, and that starting diflubenzuron levels in the uncooked fillet are at the established MRL of 1000 µg/kg would result in an estimated exposure level of

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<sup>13</sup> The EU Fish Market (<http://www.eumofa.eu/documents/20178/77960/The+EU+fish+market+-+2016+Edition.pdf>)

8.55 µg/person.

### **Comparison of estimated consumer exposure with acceptable intake levels**

In section 2.1.8 a number of approaches were presented as possible ways to determine the maximum acceptable exposure levels: (i) linear extrapolation from the BMDL5 derived from the rat carcinogenicity study to a dose associated with a cancer incidence of 1 in 10<sup>6</sup> results in a figure of 0.2 µg/person, (ii) linear extrapolation from the BMDL10 from the rat carcinogenicity study to a dose associated with a cancer incidence of 1 in 10<sup>6</sup> results in a figure of 0.35 µg/person, (iii) an approach that uses the tumour incidence at lowest tumorigenic dose as the starting point results in a figure of 2 µg/person, and (iv) the TTC approach results in a figure of 0.15 µg/person.

A comparison of the worst case consumer exposure estimate resulting from direct ingestion of 4-chloroaniline residues in fish 1 day after the end of treatment (0.76 µg/person) with the above acceptable intake levels shows that:

- 4-chloroaniline residues in fish fillet were below the acceptable limit calculated using tumour incidence at the lowest tumorigenic dose; and
- 4-chloroaniline residues in fish fillet remained above the acceptable limits derived using linear extrapolation based on BMDL5, BMDL10 and TTC up to day 5 after cessation of treatment (the worst case estimate of consumer exposure until this time was 0.6 µg/person).

As highlighted in section 3.2, regardless of the established withdrawal period, the minimum interval likely to occur between the end of treatment and slaughter is expected to be 1 week. Furthermore, residue monitoring data demonstrate that, in practice, residue levels of diflubenzuron are at least 2 orders of magnitude below the established MRL of 1000 µg/kg, indicating that 4-chloroaniline levels will not reach the above figures in practice and that the worst case exposure calculations relating to indirect exposure result in unrealistic exposure estimates.

Based on the established MRL of 1000 µg/kg the worst case exposure to 4-chloroaniline as a result of metabolism of diflubenzuron in the consumer and conversion of diflubenzuron to 4-chloroaniline during cooking is estimated as 23.55 µg/person (15 + 8.55 µg/person). This level clearly exceeds all derived acceptable limits. It is therefore proposed that, in order to ensure that consumer exposure to 4-chloroaniline remains at an acceptable level, the MRL for diflubenzuron should be reduced to 10 µg/kg. This would have the effect of reducing the worst case indirect 4-chloroaniline exposure estimates by a factor of 100, i.e. to 0.15 µg/person as a result of metabolism of diflubenzuron in the consumer and 0.0855 µg/person as a result of cooking diflubenzuron. These values are both at or below all calculated acceptable exposure limits, and when taken together remain below the acceptable exposure limit derived based on linear extrapolation of the BMDL10 value as well as the limit derived using using tumour incidence at the lowest tumorigenic dose.

It is evident from the Norwegian residue monitoring data that an MRL of 10 µg/kg is compatible with use of diflubenzuron under field conditions. The current withdrawal period for diflubenzuron-containing veterinary medicinal products is 105 degree days (based on the established MRL of 1000 µg/kg). Reducing the MRL to a new limit of 10 µg/kg will inevitably result in longer withdrawal periods, which in turn will allow for further depletion of 4-chloroaniline, assuming fish are slaughtered in accordance with the authorised conditions of use of the product.

### **3.4. Considerations on possible extrapolation of MRLs**

In line with Article 5 of Regulation (EC) No 470/2009, the CVMP is required to consider the possibility of extrapolating its recommendation on maximum residue limits in one species to other food producing

species and commodities. Based on the similarity of metabolism between salmon and other fin fish an MRL recommended in *salmonidae* can usually be extrapolated to other fin fish. However, in this case, where a metabolite is the main concern, it is not recommended to extrapolate without evidence that this metabolite is not formed in any relevant amount in every species concerned.

### **3.5. Conclusions and recommendation for the establishment of maximum residue limits**

Whereas

- the genotoxic metabolite 4-chloroaniline has been detected in salmon muscle after diflubenzuron treatment,

and having considered that:

- an ADI of 0.0124 mg/kg bw/day (744 µg per person) has been set for diflubenzuron,
- diflubenzuron is the marker residue and a ratio of marker to total residues of 0.92 has been established in salmon muscle and skin in natural proportions,
- acceptable levels of 4-chloroaniline in edible salmon tissues can be calculated by ensuring that they are associated with a cancer risk of less than 1 in 10<sup>6</sup>,
- a worst case consumer exposure estimate based on residues of 4-chloroaniline seen in salmon 1 day after the end of treatment suggests that levels of this substance could be in the region expected to be associated with a cancer risk of 1 in 10<sup>6</sup>; however, standard management and husbandry procedures as well as residue monitoring data show that, in practice, diflubenzuron residue levels in fish occur at levels that are orders of magnitude below this, in addition to which compliance with product withdrawal periods will ensure that residues of 4-chloroaniline remain at acceptably low levels,
- a 100 fold reduction in the MRL for diflubenzuron will further reduce the potential for consumer exposure to diflubenzuron and its metabolite 4-chloroaniline in commercially available fish,
- a validated analytical method for monitoring of residues of diflubenzuron has been provided with an LOQ of 1 µg/kg,

the Committee recommends that the established maximum residue limit for diflubenzuron should be modified in line with the following table:

<b>Pharmacologically active substance</b>	<b>Marker residue</b>	<b>Animal species</b>	<b>MRLs</b>	<b>Target tissues</b>	<b>Other provisions</b>	<b>Therapeutic classification</b>
Diflubenzuron	Diflubenzuron	<i>Salmonidae</i>	10 µg/kg	Muscle and skin in natural proportions	NO ENTRY	Antiparasitic agents / Agents against ectoparasites

Based on the recommended MRL the theoretical maximum daily intake (TMDI) of residues arising from the ingestion of salmon is calculated to be 3 µg/person, which represents 0.4% of the ADI.

## 4. Background information on the procedure

Request for review	7 May 2014
Steps taken for assessment of the substance	
Clock started	7 May 2014
Adoption of scientific overview and list of questions	5 June 2014
Response to list of questions submitted:	14 November 2014
Clock restarted:	17 November 2014
CVMP opinion adopted:	7 May 2015
Request for the review by the Commission	16 March 2017
Additional data received	01 September 2017
List of outstanding issues adopted	09 November 2017
Oral explanation	16 January 2018
CVMP opinion adopted:	15 March 2018