Draft Assessment Report

Evaluation of Active Substances

Plant Protection Products

Prepared according to **Regulation (EC) 1107/2009**
as it applies in Great Britain

Bixlozone (F9600)

Volume 3 – B.6 (AS)

Toxicology & Metabolism Data

Great Britain

July 2022

Version History

When	What
July 2022	Initial DAR

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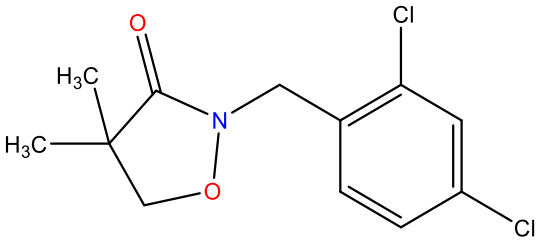
B.6. TOXICOLOGY AND METABOLISM DATA

Bixlozone (2-(2,4-dichlorobenzyl)-4,4-dimethyl-1,2-oxazolidin-3-one, also known as F9600, CAS 81777-95-9) is a new herbicidal active substance, developed by FMC Corporation (as FMC Chemical sprl). It is intended for pre- and early-post emergence application to a number of crops, including cereals, oilseed rape and maize, for control of a range of broadleaf and grass weeds.

The structure of bixlozone is presented below:



Bixlozone belongs to the isoxazolidinone chemical family and is a broadcast soil applied residual herbicide. Its mode of action is to inhibit the biosynthesis of carotenoids. After being absorbed by the roots and shoots, it is translocated upwards in water through the xylem tissue and then diffuses within the plant. Deprived of protective carotenoids, chlorophyll as well as other components of the photosynthetic apparatus becomes susceptible to photo-oxidation. Once in contact with light, these components are photodegraded and the emerging seedlings of express bleaching symptoms and die.

The representative product for bixlozone is F9600-4 SC which contains 410 g a.s./l of the active substance bixlozone. It acts as a carotenoid biosynthesis inhibitor causing bleaching of weeds. It is intended to be used as a selective herbicide for the control of annual monocotyledonous and dicotyledonous weed species in agricultural crops. The product will be applied after sowing but pre-emergence to winter wheat, winter barley, winter oilseed rape and maize or early post-emergence to winter wheat. Bixlozone does not appear to demonstrate downward systemic action or upward translocation from leaf to leaf. This may account for the inability to control larger weeds post-emergence, as well as explaining the appearance of chlorotic symptoms on contacted foliage with minimal or no effect on subsequent new growth.

This document uses the term ‘bixlozone’ when referring to the active substance. However, the development code F9600 has been used by the applicant within the individual study reports. The batches of bixlozone used in the toxicology studies are considered representative of the technical specification (see Vol 4 for more details).
The majority of the methods of analysis for the active substance in different matrices (diet, air, gavage solutions) used in the *in vivo* toxicological studies are either validated or fit for regulatory purposes (see document CA B5 and the individual studies within this B6 document for further details).

The classification of bixlozone for Human Health effects has been addressed in an aligned MCL (Mandatory Classification and Labelling) dossier produced by HSE.

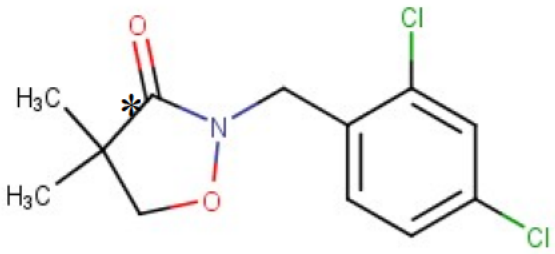
The data requirements of Regulation (EC) 1107/2009 and Regulation (EU) 283/2013 have been met (with the exception of genotoxicity data on a groundwater metabolite) and HSE concludes that there are no data gaps.

B.6.1. ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION IN MAMMALS

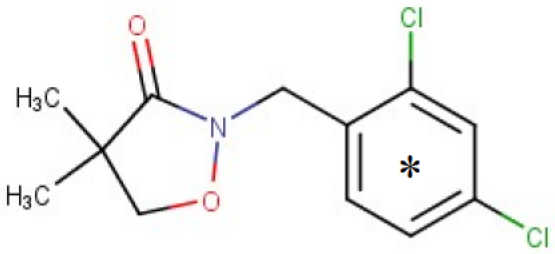
The absorption, distribution, metabolism and excretion (ADME) of bixlozone in mammals has been mainly investigated in Sprague-Dawley (SD) rats. Two different radiolabels were used in the ADME studies; bixlozone was labelled in the ^{14}C-Carbonyl of the isoxazolidinone ring or ^{14}C-Phenyl position as shown in Figure B.6.1.1 below.

Figure 6.1.1: Radiolabelled compounds of bixlozone used in rat ADME studies

[^{14}C-Carbonyl]-bixlozone



[^{14}C-Phenyl]-bixlozone



Single oral administrations included doses of [^{14}C-Phenyl]-bixlozone at 5 and 25 mg/kg bw for low doses and 500 and 1000 mg/kg bw for high doses. For repeated dosing [^{14}C-Phenyl]- bixlozone was administered at 5 mg/kg bw/day for low dose and at 500 mg/kg bw/day for high dose, over 14 days. A single oral low dose of [^{14}C-Carbonyl]- bixlozone at 5 mg/kg bw was administered to investigate the excretion routes and metabolism of [^{14}C-Carbonyl]- bixlozone in rats. Additional limited toxicokinetic information from repeated dose and carcinogenicity studies conducted in rats, mice and dogs is available and is also included in this Section of the DAR.

Furthermore, a single intravenous (IV) dose of [^{14}C-Phenyl]-bixlozone of 3 mg/kg bw was administered to male and female rats to determine the systemic availability of bixlozone. The mass balance of [^{14}C-Phenyl]-bixlozone in male bile duct cannulated rats was also assessed following a single IV dose of 3 mg/kg bw.

In addition to the *in vivo* studies available in this dossier, two comparative *in vitro* metabolism studies of bixlozone using cryopreserved hepatocytes of rats, mouse, dog and human are also available and are presented in this Section.

HSE considers these studies to provide a thorough understanding of the ADME properties of bixlozone in experimental animals following oral dosing.

B.6.1.1. Absorption, distribution, metabolism and excretion by oral route

The absorption, distribution, metabolism, elimination and plasma kinetics of bixlozone by oral route were investigated extensively in male and female rats. The studies used bixlozone radiolabelled in the ^{14}C-Phenyl or the ^{14}C-Carbonyl position respectively. Additional toxicokinetics data are also available in rats, mice and dogs from several repeated-dose toxicity and carcinogenicity studies. For further details please refer to Sections B.6.3 & B.6.5.

Pilot studies have been included in this Section however only brief summaries are available for these studies.

B.6.1.1.1. *Pilot studies*

Two non-GLP preliminary pilot studies are available.

Study 1

The objectives of this pilot study were to develop the analytical methods for the identification and quantification of bixlozone and its potential metabolites in Sprague-Dawley (SD) rat blood, urine and faeces following administration of a single oral dose of unlabelled bixlozone at 25 mg/kg bw, but also *in vitro* using rat liver microsomes. Only a brief summary of this study is presented below.

Study # 1	Pharmacokinetics and metabolism of F9600 in male and female Sprague-Dawley rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014)
Date performed	November 2013
Test facility	[redacted]
Report reference	Report no. FMC-R2838
Guideline(s)	N/A
Deviations from the guideline	N/A
GLP	No
Test material	F9600 technical; Batch G3773-17 Purity 99.5 %
Method of analysis	*The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to the lack of explanation of the use of a non-linear calibration.* However, the method is considered sufficient for regulatory purposes.
Study acceptable	Yes, but as supplementary information only since the study's objectives were to develop analytical methods for the identification and quantification of F9600 and its potential metabolites in rat blood, urine and faeces and in rat liver microsomes.

Material and Methods

Male and female rats (n = 3 per sex), fitted with a single catheter in the jugular vein for blood collection, were administered 25 mg/kg bw of unlabelled bixlozone via oral gavage. Blood samples were collected at 8 different time points over 24 hrs (30 min, 1, 2, 4, 6, 8, 10 and 24 hrs). Plasma samples and dosing solutions were analysed by LC/MS/MS using a method sufficient for regulatory purposes. The measured concentrations of the analyte were used to obtain the toxicokinetics (TK) parameters using Phoenix® WinNonlin® software.

Several references standards of potential metabolites were used alongside bixlozone in this study:

Compound	Batch number	Molecular Weight	Purity (%)
bixlozone	G3773-17	274	99.5
2,4-Dichlorobenzyl alcohol	N/A	176	N/A
2,4-Dichlorobenzaldehyde	N/A	174	N/A
2,4-Dichlorobenzoic acid	N/A	190	N/A

Urine and faeces were collected over 0-24 hr time period post dose at room temperature, and the homogenates were analysed by High-performance liquid chromatography/Ultraviolet/Mass Spectrometry (HPLC/UV/MS) after extraction with organic solvent or were directly injected.

A pilot *in vitro* metabolism study using rat liver microsomes was conducted to facilitate the identification of metabolites in biological samples. bixlozone (20 µM) was incubated in the presence of male rat liver microsomes (1 mg/mL), NADPH (2 mM) or NADPH-regenerating system (glucose-6-phosphate (3.6 mM), $NADP^+$ (1.3 mM), and glucose-6-phosphate dehydrogenase (0.4 units/mL)), $MgCl_2$ (10 mM), and UDPGA in 0.1 M phosphate buffer (pH 7.4) at 37°C for up to 1 hour in duplicates. The metabolic reaction was initiated by the addition of bixlozone after a pre-incubation period of 5 min at 37°C. At the end of the reaction period, proteins were precipitated, and samples centrifuged. The supernatants were dried and reconstituted in 20 % methanol in water for LC/UV/MS analysis. The positive control used was testosterone at 100 µM to demonstrate CYP450 activities, and was incubated under similar conditions for 30 min.

Results

Pharmacokinetics findings

Following a single oral dose of 25 mg/kg bw the half-life ($T_{1/2}$) of bixlozone was determined at 3.11 hours for males and 1.94 hours for females. The mean time to C_{max} (T_{max}), maximum plasma concentration (C_{max}) and the infinite area under the curve (AUC_{inf}) for males were 0.50 hours, 169 ng/mL and 590 hours×ng/mL, respectively; for females the mean T_{max}, C_{max} and AUC_{inf} were 0.67 hours, 315 ng/mL and 982 hours×ng/mL, respectively. Thus, the systemic exposure to bixlozone as measured by C_{max} and AUC_{inf} was approximately 1.7 to 1.9-fold greater for females in comparison to males following administration of equivalent oral doses of bixlozone.

In vitro metabolism of bixlozone in rat liver microsomes

Bixlozone appeared to be extensively metabolised in presence of rat liver microsomes. The major compound related components in the microsomal extract appeared to be isoxazolidinone ring-opened derivatives. In addition, hydroxylated metabolites were also observed however at relatively low levels.

Metabolic profile of bixlozone in rats' urine and faeces

Bixlozone appeared to be well absorbed and extensively metabolised following oral administration to male and female rats. Bixlozone was not found in urine samples; several metabolites were identified including several isoxazolidinone ring-opened/modified analogues and Phase II metabolites formed from glucuronidation of Phase I metabolites. There were no apparent differences in urinary metabolite profiles between male and female rats.

Bixlozone was also not detected in rat faeces; the metabolites observed in this matrix included isoxazolidinone ring-opened derivatives and hydroxylated metabolites. As observed with urine samples, the metabolite profiles of bixlozone appeared to be similar between male and female rats.

Conclusion

This pilot study's objectives were to develop analytical methods for the identification and quantification of bixlozone and its potential metabolites in the blood, urine and faeces of male and female rats administered 25 mg/kg bw of bixlozone via oral gavage. The $T_{1/2}$ for bixlozone 3.11 hours for males and 1.94 hours for females. It was shown that the systemic exposure to bixlozone as measured by C_{max} and AUC_{inf} was approximately 1.8-fold greater for females in comparison to males following equivalent oral doses of bixlozone.

Bixlozone appeared to be well absorbed and extensively metabolised following oral administration. Bixlozone was not found in urine or faeces samples and there were no apparent differences in urinary or faecal metabolite profiles between male and female rats. Based on the identities of the metabolites found in urine and faeces, it is proposed that the dimethylisoxazolidin-3-one moiety of bixlozone was the most susceptible site of metabolism in rats. A combination of various metabolic reactions (oxidation, ring-scission, decarboxylation) on this moiety leads to various metabolite structures, including oxidative ring-opened analogues and ring cleaved analogues. The phase I metabolites, produced by various metabolic pathways, were subsequently conjugated with glucuronic acid and excreted as glucuronides in urine. The metabolites found in faeces were primarily unconjugated and could have been derived from hepatic and/or intestinal metabolism of bixlozone.

[redacted] (2014)

Study 2

A pilot study was conducted in male Sprague-Dawley rats to investigate the excretion routes, mass balance and the metabolite profiles in urine and faeces of [^{14}C-Phenyl]-bixlozone following a single high oral dose (gavage) at 1000 mg/kg bw. The findings from this study were used to design and conduct a definitive ADME study with [^{14}C-Phenyl]-bixlozone. Only a brief summary of this study is presented below.

Study # 2	Metabolism of [^{14}C-phenyl]F9600 in male Sprague-Dawley rats - Pilot study
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2017a)
Date performed	November 2014
Test facility	[redacted]
Report reference	Study no. FMC-R3694
Guideline(s)	OECD 417 (2010)
Deviations from the guideline	N/A
GLP	No
Test material	[^{14}C-phenyl]-F9600 Batch CFQ42017; purity 99.6 %; specific activity 56 mCi/mmol (202.9 µCi/mg) Non-radiolabelled F9600 Batch PL14-0163; purity 99.8 %
Study acceptable	Yes, but as supplementary information only since this is a pilot study.

Material and Methods

Two male Sprague-Dawley rats were administered a single oral gavage dose of [^{14}C- phenyl]-bixlozone (200 µCi/kg) at 1000 mg/kg bw. After dosing, the animals were placed in individual metabolism cages with airtight enclosure under controlled air-flow and air washing systems to collect expired [^{14}C]CO_2, urine, and faeces. Urine and faeces were collected for up to 5 days post-dose whist expired air was collected for up to 48 hours post-dose. Blood was collected at the termination of the study (120-hour post-dose).

The total radioactivity of duplicate aliquots of plasma, urine, faeces and in the expired [^{14}C]CO_2 were determined by Liquid Scintillation Counting (LSC) or combination of combustion and LSC analysis. The characterisation of metabolites and their profile in pooled urine and faeces were obtained using HPLC/UV/β-RAM and LC/MS/MS using the following synthetic reference standards: bixlozone, keto-hydrate-bixlozone, bixlozone-3-OH-propanamide, 2,4-dichlorobenzoic acid, dichlorobenzyl alcohol, 3'-OH-bixlozone, bixlozone-dimethyl malonamide, bixlozone-isobutyramide, 6'-OH-bixlozone, 2,4-dichlorobenzaldehyde, 5-keto-bixlozone, 5-OH-bixlozone, 2,4-dichlorohippuric acid.

Results

Radioactivity concentrations in plasma, urine, faeces, expired air (CO_2) and cage wash

The mean total radioactivity found in plasma at 120-hour post-dose was 1.39 µg Eq/mL. The mean total recovery of radioactivity in excreta was 95 % in the 5 days post-dose. Urinary excretion, including cage wash, accounted for 72 % of the dose, and faecal excretion accounted for 24 % of the dose. A negligible amount, less than 0.1 % of the dose, was recovered in the expired air.

Furthermore, excretion was rapid, with total of 90 % of the dose was recovered within 48 hr post-dose. Urinary radioactivity accounted for 68 % of the dose and the radioactivity recovered in faeces accounted for 22 % of the dose within 48 hours post-dose.

Metabolite profile in urine

At least 13 radioactive peaks were detected. Unchanged bixlozone was not detected in urine, indicating the compound was extensively metabolised following oral dosing to rats. Two radioactive peaks in urine sample chromatograms >10 % of the dose were identified as 2,4-dichlorohippuric acid (10 %) and 5-keto-hydrate bixlozone (30 %). The remaining radioactive peaks in the urine samples were minor metabolites.

Metabolite profiles in faeces

At least 8 radioactive peaks were observed in faecal metabolite profiles. The unchanged bixlozone represented about 3 % of the dose. The radioactive peak identified as 5-OH-bixlozone, constituted about 7 % of the dose. The remaining radioactive peaks were minor metabolites, each representing individually less than 3 % of the dose.

Conclusion

In a pilot study a single oral gavage dose of [^{14}C-Phenyl]-bixlozone was administered at 1000 mg/kg bw to male rats. About 95 % of the radioactive dose was excreted in urine and faeces within 5 days following dosing. Urinary excretion accounted for about 72 % of the dose, whilst about 24 % of the dose was excreted in faeces. The radioactive dose recovered in expired air was negligible.

It was found that bixlozone was extensively metabolised. Oxidation and ring-opening, followed by conjugation constituted the major metabolic reactions observed for bixlozone in male rats. The major metabolites identified in urine were 2,4-dichlorohippuric acid (10 %) and 5-keto-hydrate bixlozone (30 %) and 5-keto-hydrate bixlozone was the most abundant metabolite in faeces (7 %).

[redacted] (2017a)

B.6.1.1.2. *Toxicokinetics and absorption studies conducted with [^{14}C-Phenyl]-bixlozone*

In this GLP and OECD guideline compliant study the toxicokinetics parameters of [^{14}C-Phenyl]-bixlozone were determined in male and female Sprague-Dawley rats following an oral low (*per os*, PO; 5 mg/kg bw) and high (1000 mg/kg bw) dose to investigate potential saturation effects. Repeated low dose (5 mg/kg bw/day, 14 days) treatment was also employed to investigate potential accumulation whilst a single intravenous dose (IV; 3 mg/kg bw) was administered to obtain information on the kinetics of bixlozone under 100% bioavailability conditions.

Study # 3	Pharmacokinetics of [^{14}C-Phenyl]F9600 in Male and Female Sprague-Dawley Rats Following Single, Multiple Oral and Intravenous Bolus Doses
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016)
Date performed	April 2016
Test facility	[redacted]
Report reference	Study no. FMC-P3773
Guideline(s)	OECD 417 (2010)
Deviations from the guideline	Some deviations from protocol occurred during the study: 1. 3 out of 4 male rats from group 7M (single IV dose) weighed slightly over 400 g (from 404 to 414 g) at the time of dosing. 3. Group 4F: 4 of 8 female rats (238 - 249 g) were outside protocol-defined range (250 - 350 g). 2. For group 4FA (single dose at 1000 mg/kg bw), animals were observed to be in distress following the 8 hours blood sampling; therefore the 10 hour blood sampling was omitted. The animals recovered by 24 hours post-dose and blood sampling was resumed for the 24 hour time point. 4. Group 1M and 2F: For the 8-hour time point, whole blood aliquots were collected but not removed prior to centrifugation; hence all blood samples were centrifuged to plasma. 5. Group 6F: Samples were not taken from rat #33F at 6 and 8-hour time points as described in protocol amendment (FMC-3773A3). 6. Group 6F: Rat #33F blood samples were taken outside the ± 30 minute time window as described in protocol at 24, 48, 72, and 96 hours. 7. Different injection volumes (3 to 10 µL) were used for plasma samples analysis for different dose groups while the injection volume stated in the validation report was 5 µL. The reason for injecting volumes adjustment was due to the variability of instrument sensitivity at different time period for the same instrument or for the different instruments of the same model used in the study. Overall HSE considers that the deviations listed above had no major effects on the outcome of the study results and should not have a significant impact on the validity of the study.

GLP	Yes. Minor shortcomings were highlighted during the study audit; however the study is considered acceptable for regulatory purposes.
Test material	[^{14}C-Phenyl]-F9600 Batch CFQ42017; purity 99.6 %; specific activity 56 mCi/mmol (202.9 µCi/mg) F9600 Batch PL 14-0163; purity 99.9 % (by HPLC)
Study acceptable	Yes, with minor deviations not considered to impact on the overall validity of the study.

Material and Methods

In this GLP and OECD Guideline compliant study (with acceptable deviations from the study protocol) [^{14}C-Phenyl]-bixlozone was administered to male and female Sprague-Dawley rats with an oral gavage low (*per os*, PO; 5 mg/kg bw) and high (1000 mg/kg bw) doses, repeated dose (5 mg/kg bw/day, 14 days) and single intravenous dose (IV; 3 mg/kg bw). The target radioactivity dose was about 7.4 MBq/kg (200 µCi/kg) for both PO and IV administrations. For repeated dosing the unlabelled bixlozone was administered to rats daily for 13 days before the last administration (14th day) was made using a radiolabelled dose of [^{14}C-Phenyl]-bixlozone.

Before the test substance administration, all rats were surgically prepared for serial blood samples collection by performing jugular vein catheterisation. The dosing design is presented in the table below:

Group	Group Number	Sex	N of Rats	Dose route & regimen		Target Dose Concentration and Volume*				
				Route	Regimen	µCi/kg	mg/kg bw	mg/mL	µCi/mL	mL/kg
Single oral low dose	1M	Male	4	PO	Day 1	200	5	0.5	20	10
	2F	Female	4	PO	Day 1	200	5	0.5	20	10
Single oral high dose	3M	Male	8	PO	Day 1	200	1000	100	20	10
	4F	Female	8	PO	Day 1	200	1000	100	20	10
Multiple oral low dose	5M	Male	4	PO	Day 1 - 14	200	5	0.5	20	10
	6F	Female	4	PO	Day 1 - 14	200	5	0.5	20	10
IV dose	7M	Male	4	IV	Day 1	200	3	1	67	3
	8F	Female	4	IV	Day 1	200	3	1	67	3

*Groups 1, 2, 3, 4, 7, and 8 were administered single doses. Groups 5 and 6 were administered multiple oral doses; non-radiolabelled bixlozone was dosed on days 1 to 13 and [14C-Phenyl]-bixlozone was dosed on day 14.

Blood samples were collected from all groups at defined time intervals. The time points for blood samples were 0.083 hour (for IV groups only), 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24, 48, 72, 96, 120, 144, and / or 168 hours post dose (n = 4 animals for each time point). For all groups except 3M and 4F (single oral high dose at 1000 mg/kg bw), blood samples were collected for up to 96 hours post-dose from 4 animals/sex/group. For groups 3M and 4F a total of 8 rats were dosed for sampling and were subsequently divided in two sub-groups (A and B, n = 4 animals for each sub-group). Sub-group A was used for blood sampling time points from 0.083 up to 96 hours, whilst sub-group B was used for blood collection from 120 up to 168 hours.

Blood and plasma samples were analysed for total radioactivity (bixlozone and its related metabolites) by liquid scintillation counting (LSC) whilst unchanged bixlozone was measured in plasma using a validated LC/MS/MS method.

Pharmacokinetic calculations of total radioactivity (plasma and blood) and unchanged bixlozone (plasma) were performed using non-compartmental analyses. For single oral doses, the pre-dose concentrations of total radioactivity and unchanged bixlozone were set to zero. For IV dosing, the total radioactivity and bixlozone concentrations at time zero (C_0) were obtained by extrapolation to time zero.

For single oral doses C_{max} and T_{max} were obtained directly from the raw data as the coordinates of the highest concentration of the time course.

C_{max} and AUC values of total radioactivity and bixlozone were also normalised to the dose.

For the 5 mg/kg bw dosing, the total radioactivity and bixlozone accumulation ratios either based on C_{max} (R_AC_{max}) or on $AUC_{0\text{-}inf}$ ($R_AAUC_{0\text{-}inf}$) were calculated as follows:

$$R_AC_{max} = \frac{C_{max}\ Day\ 14}{C_{max}\ Day\ 1} \quad \& \quad R_AAUC_{0\text{-}inf} = \frac{AUC_{0\text{-}inf}\ Day\ 14}{AUC_{0\text{-}inf}\ Day\ 1}$$

Bioavailability (F) of bixlozone and its metabolites, and of bixlozone only were calculated according to the following equation based on individual and mean values:

$$\%\ F = 100 \times \frac{AUC_{0\text{-}inf,\ PO} \times Dose_{IV}}{AUC_{0\text{-}inf,\ IV} \times Dose_{PO}}$$

Plasma and blood concentrations of bixlozone as well as toxicokinetics parameters of total radioactivity were tabulated according to the dose, route of administration and sex. For IV and oral doses, plasma bixlozone to total radioactivity C_{max} and $AUC_{0\text{-}inf}$ individual ratios were also reported.

Results

*Radioactivity in blood and bixlozone plasma concentrations (**Error! Reference source not found.**)*

Female rats had a relatively higher mean total radioactivity than male rats in both whole blood and plasma following single or repeated oral doses. The mean blood to plasma ratios were between 0.5 to 1.0 within 24 hour post-dose for all dose groups indicating that [^{14}C-Phenyl]-bixlozone and related components had limited partitioning into red blood cells. The ratios appeared to increase after 48 hours; however no clear bioaccumulation was observed.

The maximum plasma concentrations occurred between 0.25 and 4 hours after oral administration. Following a single or repeated oral dose of bixlozone at 5 mg/kg bw, the mean plasma concentrations decreased quickly over time and no bixlozone was detected in plasma 10 hours post-dosing. A less than proportional internal exposure to dose was observed in both sexes when the oral dose was increased from 5 to 1000 mg/kg bw, suggesting non-linear kinetics in the rat at high doses, presumably as a consequence of saturation of absorption. Bixlozone concentrations were close to the lower limit of quantification by 96 hours post-dosing following the oral high dose.

Bixlozone plasma concentrations are presented in the table below:

Table B 6.1.1.1: Plasma concentrations of bixlozone after single and repeated oral administration or single IV injection of [^{14}C-Phenyl]-bixlozone to male and female rats

Time Point (h)	Bixlozone concentrations in Plasma (ng/mL)*							
	Single oral low dose 5 mg/kg bw		Single oral high dose 1000 mg/kg bw		Repeated oral low dose 5 mg/kg bw/day		Single IV dose 3 mg/kg bw	
	Group 1M	Group 2F	Group 3M	Group 4F	Group 5M	Group 6F	Group 7M	Group 8F
0.083	N/A	N/A	N/A	N/A	N/A	N/A	1317±272	1195±115
0.25	174±109.6	293±74.1	4065±312	7008±1773	70.6±15.9	165±127	667±113	730±106
0.5	80.7±34.91	125±21.9	5630±1351	8168±3757	35.0±10.3	93.2±44.8	373±52.9	360±36.6
1	40.2±14.56	50.5±6.68	7695±1740	10713±6063	18.5±8.3	44.9±19.0	138±17.3	115±14.2
2	16.3±6.83	21.4±5.35	9423±2849	12488±7391	6.71±2.1	15.6±4.8	56.3±12.0	50.9±6.7
4	5.47±2.74	8.22±1.74	9465±2683	14535±10643	2.73±0.8	7.25±2.9	20.5±3.8	20.2±3.3
6	1.79±0.78	3.47±1.22	6818±2791	13425±9863	1.12±0.8	3.28±1.2	9.93±0.6	11.1±2.5
8	1.22±0.34	2.47±1.07	4998±2861	13958±11017	1.04±0.09	2.44±0.9	7.37±0.2	N/A
10	1.28±0.56	1.05±0.11	3288±2488	N/A	1.11±0.21	1.80±0.8	5.98±1.7	18.1±25.9
24	< BQL	< BQL	627±446	1951**	< BQL	< BQL	6.35±10.7	< BQL
48	< BQL	< BQL	63.0±34.7	166±46.6	< BQL	< BQL	3.11±3.69	< BQL
72	< BQL	< BQL	11.1±12.1	17.0±9.03	< BQL	< BQL	2.26±2.51	N/A
96	< BQL	< BQL	2.17±0.88	6.13±4.84	< BQL	< BQL	< BQL	1.03±0.06
120	N/A	N/A	1.32±0.40	2.35±0.89	N/A	N/A	N/A	N/A
144	N/A	N/A	< BQL	1.65±0.94	N/A	N/A	N/A	N/A
168	N/A	N/A	< BQL	1.27±0.32	N/A	N/A	N/A	N/A

* The values that were below the quantification limit (BQL value = 1 ng/mL) were treated as the worst case value of 1 ng/mL for the calculation of the means and standard deviation values.
** 2/4 concentrations were over above the upper limit of quantification (ULQ)with 20-fold dilution. Since insufficient sample volume was available for further dilution; the values above the ULQ was not be used for PK calculation and the mean was calculated with n = 2 only.
< BQL: all values were below the BQL for these endpoints

Pharmacokinetics parameters for total radioactivity and unchanged bixlozone in plasma (Table B 6.1.1.2 & Table B 6.1.1.3)

Based on the AUC values calculated for each group, the systemic exposure to total radioactivity was slightly higher in female rats compared to the male rats at equivalent doses. The ratio between the AUC values and the dose levels (5 vs 1000 mg/kg bw) was proportional for male rats but was sub-linear for females, indicating a reduction in the oral absorption of bixlozone with increasing dose levels in females.

Oral absorption was rapid following single or repeated oral low dose (5 mg/kg bw) with C_{max} reached in less than an hour post-dosing for both sexes. Oral absorption was slower following oral high dose (1000 mg/kg bw), with C_{max} reached between 15-24 hours post-dosing.

The systemic bioavailability of bixlozone and/or its metabolites (expressed as F and comparing the AUC_{oral} with the AUC_{IV}) was at 70% in males and 86% in females respectively for the single oral low dose groups (groups 1M & 2F, 5 mg/kg bw), indicating high oral absorption and systemic availability at the low dose. Bioavailability was lower following a single oral high dose (groups 3M & 4F, 1000 mg/kg bw), with respective values at 58% and 60% in the male and female groups (3M & 4F groups).

Regarding the kinetics parameters for the unchanged bixlozone, it is noted that bixlozone was rapidly cleared from plasma, with $T_{1/2}$ of less than 2 hours for the IV and oral low dose groups. At the high dose, a notable increase in $T_{1/2}$ was observed (11-14 hours), indicating a more prolonged, slower absorption. Following repeated oral low dosing with bixlozone at 5 mg/kg bw/day (groups 5M & 6F), no accumulation of unchanged bixlozone was observed in plasma at the end of 14 days since the accumulation ratios expressed as R_AC_{max} and R_A $AUC_{0\text{-}inf}$ and based on C_{max} and $AUC_{0\text{-}inf}$ were both < 1 for both sexes.

The bioavailability (F % values of 11 and 18% in males and females, respectively) and the systemic plasma exposure of bixlozone as measured by $AUC_{0\text{-}inf}$, were approximately 1.5-fold greater in low dose females compared to males.

Comparison of plasma concentrations of bixlozone and/or its metabolites (total radioactivity) and unchanged bixlozone from IV dosing showed that unchanged bixlozone in plasma accounted for 27 % to 39 % of total radioactivity at the earliest time point examined. However, plasma $AUC_{0\text{-}inf}$ of bixlozone accounted for only about 2 % to 3 % of total radioactivity, in male and female rats. These data indicate that extensive metabolism of bixlozone occurs in rats. In the single oral dose groups (1M, 2F, 3M & 4F), plasma $AUC_{0\text{-}inf}$ of unchanged bixlozone accounted for about 0.5% (5 mg/kg bw) and 2 to 6% (1000 mg/kg bw) of bixlozone-related total radioactivity in plasma in both male and female rats. The low systemic levels of bixlozone, as compared to total radioactivity, suggested significant metabolism of this compound in male and female rats.

The mean kinetics parameters for total radioactivity and unchanged bixlozone in plasma are presented in the tables below:

Table B 6.1.1.2: Plasma kinetic parameters of bixlozone and its metabolites (total radioactivity) after a single / repeated oral administration or a single IV injection of [^{14}C-Phenyl]-bixlozone to male and female rats

Group	C_{max} ng/mL	T_{max} h	t_{last} h	$AUC_{0\text{-}last}$ ng×h/mL	$T_{1/2}$ h	$AUC_{0\text{-}inf}$ ng×h/mL	F %	R_AC_{max}	$R_AAUC_{0\text{-}last}$
1M	4.71 ±1.5	0.38±0.14	78±12	27 ±4	15.4 ±5.9	28±4	70		
2F	6.80 ±0.5	0.25±0.00	96±0	46±5.8	15.4±4.1	46±5.8	86		
3M	141±47.0	15.5±10.1	90±12	4526±1314	13.0±1.4	4575±1317	58		
4F	178±135	24±0.0	96±0	6304±3425	16.3±4.1	6363±3427	60		
5M	3.91±1.1	0.31±0.1	66±12	22±4.3	10.7±1.4	23±3.9	58	0.83	0.82
6F	3.83±0.9	0.75±0.8	66±30	40.0±8.6	11.2±1.4	43.0±5.5	79	0.56	0.93
7M	3.53±0.2	0.17±0.10	96±0	24±0.9	16.2±7.5	24±1.0	-		
8F	4.37±0.4	0.25±0.00	96±0	32±2.2	11.6±0.5	32.0±2.2	-		

C_{max}: maximal concentration; T_{max}: time to reach maximal concentration; $t_{1/2}$: terminal half-life; $AUC_{0\text{-}last}$: Area under the concentration-time curve up to the last detectable concentration; $AUC_{0\text{-}inf}$: Area under concentration-time curve up to infinite time; F: bioavailability; R_AC_{max}: Accumulation ratios, based on C_{max} on day 14 and day 1; RA $AUC_{0\text{-}inf}$: Accumulation ratios, based on $AUC_{0\text{-}inf}$ on day 14 and day 1.

M: males; F: females.

Table B 6.1.1.3: Plasma kinetic parameters of bixlozone after single / repeated oral administration or single IV injection of [^{14}C-Phenyl]-bixlozone to male and female rats

Group	C_{max} ng/mL	T_{max} h	t_{last} h	$AUC_{0\text{-}last}$ ng×h/mL	$T_{1/2}$ h	$AUC_{0\text{-}inf}$ ng×h/mL	F %	R_AC_{max}	$R_AAUC_{0\text{-}last}$
1M	174±110	0.25±0	7±1	143±67.2	1.38±0.3	145±67.5	11±5		
2F	293±74.1	0.25±0	9±1	217±40.6	1.71±0.2	221±40.3	18±3		
3M	9565±2832	3.5±1	108±24	105550±37052	10.99±0.9	105678±37241	39±13.9		
4F	15060±10362	3.5±1.9	150±23	358519±296742	13.9±3	358536±296749	141±117		
5M	70.6±15.9	0±0	6±2	61.0±19.1	1.71±0.4	65.0±19.4	5±1.4	0.41	0.45
6F	165.5±127.4	0.25±0	8±3	153±66.2	2.09±0.8	162±67.5	13±5.6	0.56	0.73
7M	1317±272.1	0.08±0	10±0	790±127	2.04±0.5	801±128.5			
8F	195±115	0.08±0	9±2	740±79.3	2.66±0.8	761±77.8			

* Calculated value was 141; C_{max}: maximal concentration; T_{max}: time to reach maximal concentration; t_{last}: time of the last quantifiable concentration; $T_{1/2}$: terminal half-life; $AUC_{0\text{-}last}$: Area under the concentration-time curve up to the last detectable concentration; $AUC_{0\text{-}inf}$: Area under concentration-time curve up to infinite time; F: bioavailability; R_AC_{max}: Accumulation ratios, based on C_{max} on day 14 and day 1; RA $AUC_{0\text{-}inf}$: Accumulation ratios, based on $AUC_{0\text{-}inf}$ on day 14 and day 1
M: males; F: females.

Conclusion

In this GLP and OECD compliant study the pharmacokinetics parameters of [^{14}C-Phenyl]-bixlozone following a single IV (3 mg/kg bw), oral low (5 mg/kg bw) and oral high (1000 mg/kg bw) dose, and repeated dose (5 mg/kg bw/day, 14 days) were determined in male and female Sprague-Dawley rats.

Bixlozone was rapidly and highly absorbed following a low oral administration to male and female rats and its clearance from the blood compartment was fairly rapid ($T_{1/2} < 2$ hours). The bioavailability of bixlozone and/or its metabolites in plasma of male and female rats was 70% and 86% in the single low dose groups, and lower at 58% and 60% in the single high dose groups, respectively. Less than proportional internal exposure to dose was observed in both sexes when the oral dose was increased, suggesting non-linear kinetics in the rat at high doses, presumably as a consequence of saturation of absorption.

In general, bioavailability of bixlozone was higher (1.5-3 fold) in females than in males at the same dose level. No accumulation of total radioactivity and unchanged bixlozone was observed in plasma following repeated dosing (14 days). The low post-hepatic exposure to unchanged bixlozone, as compared to total radioactivity, suggested significant metabolism of this compound in male and female rats.

[redacted] (2016)

B.6.1.1.3. *Complementary toxicokinetic information from repeated dose and carcinogenicity studies*

Complementary information on toxicokinetics is available from several repeated dose studies on bixlozone. The plasma levels of bixlozone were determined in rat and mouse chronic/carcinogenicity studies, in the 28-day rat study and in the 90-day mouse and dog studies. Short summaries of the findings are presented below. The methods of analysis for these plasma measurements were fully validated or valid for regulatory purposes. Please refer to Sections B.6.3 (repeated dose toxicity) and B.6.5 (carcinogenicity) for further details on the studies conducted.

Rat

Study 1 (28-day rat; Section ***Error! Reference source not found.)***

In a GLP and OECD-compliant 28-day dietary repeated-dose toxicity study conducted in the rat ([redacted] (2015a), the concentration of bixlozone in Sprague Dawley (SD) rats' plasma was determined following administration of bixlozone via the diet for 28 consecutive days to 9 animals/sex/group at doses of 750, 2500, 5000 and 10000 ppm. These doses equated to intakes of 57, 182, 359 & 740 mg/kg bw/d in males and 61, 193, 379 & 733 mg/kg bw/day in females. Concurrent control groups received basal diet only (3 animals/sex).

Blood was collected from 3 animals/sex in the control groups at 14:00 hours on days 1, 14 and 27. For the treated groups blood samples were collected from 3 animals/sex/group at 06:00, 10:00, 14:00, 18:00, 22:00, and 02:00 hours on study days 1-2 and 27-28, as well as at 14:00 hours on study days 7, 14, and 21. Plasma was analysed for the concentration of bixlozone, using a validated LC/MS/MS method. The pharmacokinetics parameters calculated included C_{max}, T_{max}, the area under the plasma concentration vs. time curve (AUC). An assessment of accumulation of bixlozone was calculated using the following formula: R_A AUC_{last} = AUC_{last} Day 27 / AUC_{last} Day 1.

Results *(Table B 6.1.1.4)*

The dietary administration of bixlozone to male and female rats resulted in systemic exposure to unchanged bixlozone, with plasma concentrations ranging from BLQ (< 5.00 ng/mL) to 509 ng/mL. Exposure to unchanged bixlozone increased with dose on both evaluation days for both sexes, with an exception on day 1 for females, where the plasma concentrations plateaued at 5000 and 10000 ppm, indicating possible saturation of absorption.

Exposure (C_{max}) was higher for females than males by a magnitude of 2- to 9-fold on day 1 of the study and was 5- to 31- fold higher at the end of the study (day 27). The extent of the difference between males and females was inversely proportional to the increase in dose, i.e. the sex difference was greater at lower doses and smaller at the top dose. Moreover, systemic exposure to bixlozone appeared to be lower for both sexes on study day 27 compared to study day 1 at all dose levels. Comparing systemic exposure to bixlozone to food consumption in both sexes, it was noted that food consumption was statistically significantly reduced in females from 2500 ppm whilst no change was reported in males up to the top-dose during the length of the study. Thus, this could indicate a possible increased metabolism of bixlozone related to enzyme induction or a saturation of absorption over time, resulting in lower plasma levels, with effects more pronounced in males compared to female rats.

Thus, the greater systemic exposure of females to bixlozone might explain the apparent heightened sensitivity of females (compared to males) to bixlozone-induced toxicity (reduced body weight gain, adverse liver effects observed at lower doses in females compared to males; please refer to Section B.6.3.2.1 for more details).

The T_{max} for bixlozone correlated with the expected nocturnal food consumption in the rat since all T_{max} time values on day 1 and 27 were either at 22:00, 2:00 or 6:00.

Table B 6.1.1.4: Toxicokinetics data from the 28-day rat study

Dose (ppm)	Males					Females				
	0	750	2500	5000	10000	0	750	2500	5000	10000
(mg/kg bw/day)	0	57	182	359	740	0	61	193	379	733
	AUC_{last} (h ng/mL)									
Day 1		88.1	268	909	1670		507	2030	3260	3130
Day 27		10.7	72.2	251	510		332	723	1210	2370
	C_{max} (ng/mL)									
Day 1		11.7	25.5	92.8	146		44	225	399	258
Day 27		5.36	7.24	22.8	47.6		29.5	69.7	118	242
	T_{max} (h)									
Day 1		6:00	6:00	6:00	6:00		6:00	6:00	22:00	22:00
Day 27		6:00	22:00	2:00	6:00		22:00	22:00	6:00	2:00

A lack of accumulation of bixlozone after repeated-dosing was indicated by R_A AUC_{last} values < 1; ratios ranged from 0.122 to 0.306 for males and from 0.356 to 0.757 for females from the lowest to the top dose.

Table B 6.1.1.5: Accumulation ratios (R_A AUC_{last}) for bixlozone in male and female rats from the 28-day rat study

Dose (ppm)	Sex	R_A AUC_{last}
750	M	0.122
	F	0.655
2500	M	0.270
	F	0.356
5000	M	0.276
	F	0.372
10000	M	0.306
	F	0.757

Study 2 (2-year rat; SectionB.6.5.1)

In a GLP- and OECD-compliant combined dietary chronic toxicity / carcinogenicity study ([redacted] (2017)), rats were treated with bixlozone at 250, 1000, and 5000/3000 ppm (males/females), equating to consumed levels of 10, 41, and 217 mg/kg bw/day for males and 13, 53, and 167 mg/kg bw/day for females. Blood samples from the toxicokinetic groups (4 animals/sex) were collected for 3 animals/sex/day/group between 06:00 and 08:00 hours on day 1, 14, 28, 90, and 182. For the chronic / carcinogenicity groups, blood samples were collected from all animals (10 animals/sex/day/group) on Day 363 or 364 (1 year) and Day 727, 728, 729, 730, or 733 (2 years). Plasma was analysed for the concentration of bixlozone using a LC-MS/MS method.

Results *(Table B 6.1.1.6)*

Consistent with the previous toxicokinetics findings from the 28-day repeated dose study, plasma concentrations of bixlozone increased with the dose up to the maximum dose in both sexes. The highest exposure was recorded on day 1 for both sexes. On the following sampling days, plasma concentrations were noted to be lower compared to day 1 from 1000 ppm in both sexes, and concentrations remained stable up to the end of the study. Test substance-related lower mean food consumption, which could partly explain the lower plasma concentrations of bixlozone observed after study initiation, was noted in the 3000 ppm group females and the 5000 ppm group males compared to the control group, especially during the chronic toxicity phase (0-52 weeks). Since bixlozone exposure remains higher in females than in males despite females consuming less food than males (please refer to Section B.6.5.1 for more details), it is possible that males exhibit an enhanced metabolism of the test substance over time compared to females.

Consistent with the findings from the short-term 28-day rat study, there is no indication that bixlozone accumulates upon chronic dosing.

Table B 6.1.1.6: Plasma concentrations of bixlozone in the combined chronic toxicity and carcinogenicity rat study

	N	Males			Females		
Dosage (ppm)		250	1000	5000	250	1000	4000/3000*
mg/kg bw/day		10	41	217	13	53	167
Day 1	3	6.07 ± 1.9	9.61 ± 7.4	83.4 ± 33.6	8.56 ± 3.8	45.1 ± 21.7	424 ± 181
Day 14	3	BLQ	5.31 ± 0.5	24.8 ± 5.96	7.37 ± 2.4	28.9 ± 5.71	88.1 ± 54
Day 28	3	BLQ	5.32 ± 0.4	25.0 ± 7.75	8.33 ± 5.8	22.0 ± 14.3	94.1 ± 88.4
Day 90	3	BLQ	8.99 ± 1.7	30.1 ± 11.6	11.08 ± 3.6	47.4 ± 8.59	105 ± 42.9
Day 182	3	6.27 ± 1.1	11.2 ± 4.3	22.7 ± 6.46	10.35 ± 6.5	37.2 ± 14.4	79.0 ± 15.7
Year 1	10	5.01 ± 0.04	8.13 ± 5.32	17.5 ± 7.99	7.29 ± 2.19	20.0 ± 12.7	52.8 ± 27.9
Year 2	21-23 M 12-17 F	5.96 ± 3.04	12.27 ± 9.61	34.0 ± 24.3	11.1 ± 6.0	23.7 ± 18.6	68.0 ± 54.2

N = 3.
* = For females, dosage level was lowered on Study Day 49 from 4000 ppm to 3000 ppm due to excessive toxicity.
BLQ = Below the limit of quantitation (5.00 ng/mL). For values assigned as BLQ the most conservative value used was 5.00 ng/mL to estimate the mean plasma concentration unless all samples (n = 3) were assigned as BLQ.

Mouse

Study 1 (90-day mouse; SectionB.6.5.2)

In a GLP- and OECD-compliant dietary 90-day repeated-dose toxicity study ([redacted](2016b)) doses of 0, 1000, 2250 & 5000 ppm (equivalent to 0, 180, 414 & 930 mg/kg bw/day and 0, 257, 583 &1185 mg/kg bw/day in males and females, respectively) of bixlozone were administered to Crl:CD1(ICR) mice for 90 days. For the toxicokinetics evaluation, blood samples were collected from 3 animals/sex/group on study days 1, 21, 56, and 90.

***Results** (Table B 6.1.1.7)*

Systemic exposure to bixlozone in mice was observed, which increased with dose on study day 1 for males (with a slight decrease at 1000 ppm and 2250 ppm in females as an exception). Contrary to the effect observed in rats, exposure in mice was generally higher for males than for females by a magnitude of 2- to 9-fold. Comparable to the findings in rats, the magnitude of the sex difference was inversely proportional to the dose. Exposure was generally lower on study days 21 and 56 (both sexes) and also on study day 90 (females), although there were no clear treatment-related effects observed on body weight development or food consumption in both sexes over the 90 days exposure (please refer to Section **Error! Reference source not found.** for more details). There was also no indication that bixlozone accumulates upon repeated dosing.

Table B 6.1.1.7: Plasma concentrations of bixlozone in the 90-day mouse study

	Males			Females		
Dose (ppm)	1000	2250	5000	1000	2250	5000
mg/kg bw/day	180	414	930	257	583	1185
	Mean plasma concentration ng/L					
Day 1	35.1 ± 10.4	139 ± 64.8	182 ± 51.7	48.5 ± 58.6	26.3 ± 11.9	140 ± 7.02
Day 21	48.3 ± 9.35	49.6 ± 12.2	98.6 ± 16.3	5.64 ± 0.09	18.8 ± 7.07	29.8 ± 5.87
Day 56	16.6 ± 3.49	46.8 ± 24.3	42.4 ± 14.1	8.92 ± 2.92	26.6 ± 18.5	36.6 ± 11.5
Day 90	16.5 ± 13.4	58.8 ± 39.5	45.4 ± 30.3	5.2 ± 0.3	13.9 ± 9.7	21.1 ± 12.9

Note: Blood samples were collected at approximately 06:00 hours at each interval. For values assigned as BLQ the most conservative value used was 5.00 ng/mL to estimate the mean plasma concentration unless all samples (n = 3) were assigned as BLQ.

Study 2 (18-month mouse, Section B3.5.2)

In a GLP and OECD compliant carcinogenicity study ([redacted](2017)), toxicokinetics groups of Crl:CD1(ICR) mice (20 animals/sex/group) were treated with bixlozone technical for 52 weeks at 250, 1000, and 5000 ppm for both sexes (equating to 38, 150, 756 mg/kg/day, and 50, 202, and 1046 mg/kg/day, for males and females, respectively). Blood samples were collected from 3 animals/sex/toxicokinetics group on Day 1, 14, 28, 90, 180 and 365 days.

***Results** (Table B 6.1.1.8)*

There was high variability in bixlozone concentrations among individuals of the same dose groups, with standard deviations that were near or exceeded the mean for all dose groups on most sampling days. Consistent with the findings from the 90-day study, bixlozone mean concentrations were found to be 2- to 13-fold higher in male than in female mice on most sampling days at all dose levels.

Bixlozone mean concentrations tended to be higher on days 14 and/or 28 than on other evaluation days. At day 1 the mean plasma concentrations following administration of bixlozone at 1000 ppm in males and females were similar or lower than those at 250 ppm; much higher concentrations were seen on day 14 and 21.

Table B 6.1.1.8: Plasma concentration (ng/mL) for bixlozone after dietary administration of bixlozone technical in mice

Males						
Bixlozone (ppm)	**250**		**1000**		**5000**	
mg/kg bw/day	**38**		**150**		**756**	
Study Day	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1	22.5	4.75	17.9	13.6	146	166
14	69.1	99.3	356[a]	561	173	97.2
28	13.7	5.36	63.2	36.7	209	179
90	13.4	8.68	20.1	6.42	78.1	39.4
182	15.2	9.96	33.3	16.1	115	39.8
366	10.7	5.85	42.9	21.6	144	98.6
Females						
Bixlozone (ppm)	**250**		**1000**		**5000**	
mg/kg bw/day	**50**		**202**		**1046**	
Study Day	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1	13.3	11.2	8.2	5.5	128	102
14	10.0	8.7	51.3	52.7	76.4	67.6
28	6.2	2.1	31.5	23.5	193	142
90	BLQ	-	6.5	1.5	34.5	15.5
182	BLQ	-	5.8	1.4	35.3	22.0
366	BLQ	-	8.7	3.8	37.3	29.2

N = 3

BLQ Below the limit of quantitation (5.00 ng/mL). For values assigned as BLQ the most conservative value used was 5.00 ng/mL to estimate the mean plasma concentration unless all samples (n = 3) were assigned as BLQ.

a Mean concentration included high concentration of 1000 ng/mL for 1 male; this concentration was up to 37-fold higher than concentrations in other animals in this group on Day 14. If 1000 ng/mL value is excluded, the mean plasma concentration is 32.3 (no SD with N = 2).

Dog

*Study 1 (90-day dog; Section **Error! Reference source not found.**)*

In a GLP- and OECD-compliant dietary 90-day repeated-dose toxicity study ([redacted] (2016c)), bixlozone technical was administered in capsules to males and females Beagle dogs (4 animals/sex/group) for 90 consecutive days at 30, 100, 300 and 750 mg/kg bw/day. Blood was collected from all animals 4-hours post-dose on study days 0, 38 and 89.

***Results** (Table B 6.1.1.9)*

Plasma concentrations of bixlozone increased in line with dose at the 4-hour measurement although the increases were not proportional to the dose and intra-group variability was high. For example, the 7.5-fold dose increase from 100 to 750 mg/kg bw/day resulted in a corresponding 4.3- to 110-fold increase in plasma concentration of bixlozone on all days. There was no apparent difference between males and females (aside from on study day 30 when the plasma concentrations were below the limit of quantification); the plasma concentrations observed do not indicate that there is any accumulation of bixlozone in dogs.

Table B 6.1.1.9: Plasma concentrations of bixlozone in the 90-day dog study

	Mean concentration in plasma (ng/mL)							
	Males				Females			
Dosage (mg/kg bw/day)	**30**	**100**	**300**	**750**	**30**	**100**	**300**	**750**
Day 0	7.7 ± 2.9	8.0 ± 4.0	54.6	611	BLQ	8.5 ± 4.0	26.3	355
Day 38	11.0 ± 9.9	BLQ	21.1	71.9	BLQ	12.5 ± 4.0	10.9	54.4
Day 89	BLQ	11.0 ± 9.9	15.4	42.6	BLQ	8.5 ± 4.4	15.6	32.3

BLQ Below the lower limit of quantitation. For values assigned as BLQ the most conservative value used was 5.00 ng/mL to estimate the mean plasma concentration unless all samples (n = 3) were assigned as BLQ

B.6.1.1.4. *Distribution studies conducted with [^{14}C-Phenyl]-bixlozone*

Two studies investigating the distribution of bixlozone (labelled in the phenyl ring) and/or its metabolites are available.

Study 1 (main study)

The tissue distribution of [^{14}C-Phenyl]-bixlozone was investigated in male and female Sprague-Dawley rats at the T_{max} determined from a previous pharmacokinetic study (Section B.6.1.1.2).

Study # 4	Tissue distribution of [^{14}C-Phenyl]F9600 at peak concentration (T_{max}) in male and female Sprague-Dawley rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2017b)
Date performed	September 2017
Test facility	[redacted]
Report reference	Study no. FMC-P4973
Guideline(s)	OECD 417 (2010)
Deviations from the guideline	None
GLP	Yes. Minor shortcomings were highlighted during the study audit; however the study is considered acceptable for regulatory purposes.
Test material	[^{14}C-Phenyl]-F9600 Batch 77874-3-19; purity 100 %; specific activity 56 mCi/mmol (202.9 µCi/mg) F9600 Batch PL 14-0163; purity > 99 % (by HPLC)
Study acceptable	Yes

Material and Methods

In this GLP and OECD guideline-compliant study, the tissue distribution of [^{14}C-Phenyl]-bixlozone was investigated in groups of 4 male and female Sprague-Dawley rats administered a single or repeated low (5 mg/kg bw) or a single high (500 mg/kg bw) oral gavage dose. For the repeated dose schedule, the unlabelled dose (bixlozone) was administered to rats daily for 13 days before the last administration (14th day) was made with a radiolabelled dose of [^{14}C-Phenyl]-bixlozone.

Following the administration of the doses, the animals were sacrificed at ≈ T_{max} determined from a previous study (Section B.6.1.1.2) and corresponded to 0.5 hour for the 5 mg/kg bw dose groups and 4 hours for the 500 mg/kg bw dose groups. Selected tissues and whole blood/plasma were collected from the rats. Urine, faeces and cage wash were not analysed in this study as the focus of the study was on tissue distribution.

The total radioactivity in whole blood, plasma, selected tissue (skin, brain, liver, kidneys, GI tract, lung, heart, white fat, spleen, adrenals, thyroid, testes / ovaries & uteri collected together, skeletal muscles, bone) and carcasses were determined, and the blood and tissue to plasma ratios of total radioactivity were calculated.

The % dose in tissues was calculated as the radioactivity in plasma (dpm/g) ÷ 2220000 (dpm/µCi) x tissue weight (g) ÷ total radioactivity dosed (µCi) %.

Results

Radioactivity in blood / plasma samples (Table B 6.1.1.10)

The blood to plasma ratios approximated 0.5, suggesting that bixlozone and its related components did not preferentially partition into whole blood cells. These results are consistent with the findings of the previous pharmacokinetic study (Section B.6.1.1.2).

In males, the mean radioactive concentrations found in plasma and blood increased only slightly following repeated dosing (less than 2-fold increase after 14 days) compared to those retrieved after single dosing; the values remained similar in females. These results indicate that there is comparable absorption and no saturation occurring upon repeated dosing. Moreover, as seen previously in the pharmacokinetic study (Section B.6.1.1.2), the mean radioactive concentrations found in female rats at T_{max} were about 1.7-fold higher than in male rats following a single dose; however this was not observed following repeated dosing.

At the T_{max} of the single oral high dose (500 mg/kg bw) the mean radioactivity found in plasma and blood, compared to those retrieved after a single oral low dose (5 mg/ kg bw), showed that the increase in radioactivity concentrations was not proportional to the increase in the dose (concentration increases were ≈ 35-fold for males

and 15-fold for females for a 100-fold dose increase). Furthermore, higher mean radioactivity was observed in male blood and plasma at the T_{max} compared to female rats following administration at 500 mg/kg bw. These findings indicate a possible saturation of absorption at the high dose.

The concentrations of total radioactivity found in blood and plasma as well as the blood to plasma ratios found in male and female rats following single or multiple oral doses of [^{14}C-Phenyl]-bixlozone are shown in the table below.

Table B 6.1.1.10: Mean plasma and blood concentrations of total radioactivity and blood / plasma ratios found in male and female Sprague-Dawley rats following single (low and high) and multiple (low) oral dose of [^{14}C-Phenyl]-bixlozone

Dose group	Time Point at T_{max}* (hours)	Total radioactive Concentrations (µgEq/g)		Blood/Plasma Ratios
		Plasma	Blood	
Males, single oral low dose at 5 mg/kg bw	0.5	4.27 ± 1.35	2.05 ± 0.68	0.48 ± 0.02
Females, single oral low dose at 5 mg/kg bw	0.5	6.82 ± 1.43	3.47 ± 0.61	0.51 ± 0.03
Males, repeated oral low dose at 5 mg/kg bw (day 14)	0.5	7.21 ± 1.46	3.56 ± 0.55	0.49 ± 0.04
Females, repeated oral low dose at 5 mg/kg bw (day 14)	0.5	6.03 ± 0.95	2.93 ± 0.49	0.49 ± 0.06
Males, single oral high dose at 500 mg/kg bw	4	139.31 ± 38.00	75.88 ± 22.50	0.54 ± 0.03
Females, single oral high dose at 500 mg/kg bw)	4	99.54 ± 32.27	57.70 ± 20.36	0.58 ± 0.03

* The 0.5 and 4 hour time points corresponded to the T_{max} values for low and high dose groups as specified in the guideline for collecting tissues

Radioactivity in tissues and carcasses (Table B 6.1.1.11, Table B 6.1.1.12 & Table B 6.1.1.13)

Similar radioactivity levels were found in the residual tissues and carcasses of male and female rats administered single or multiple low (5 mg/kg bw) oral doses (Table B 6.1.1.11Table B 6.1.1.11). The data show that [^{14}C-Phenyl]-bixlozone was rapidly and widely distributed in rats at T_{max} after either single or repeated oral administration. The highest levels of radioactivity (in decreasing order) were found in the gastrointestinal tract (GI tract; 27-38 µgEq/g) followed by the liver (8-12 µgEq/g), kidney (3-6 µgEq/g) adrenals (2-6 µgEq/g) and thyroid (1-7 µgEq/g); lower levels were found in white fat, lung, blood, ovaries and uteri, heart, spleen, skin, brain, muscles, bone, testes, and the residual carcass.

The majority of the dosed radioactivity (≈ 80 %) was recovered in the tissues collected (Table B 6.1.1.12). Among all the tissues analysed, the GI tract (≈ 50-65 % of dose), liver (≈ 1.5-8 % of dose) and blood (≈ 0.3-1.8 % of dose) held the highest % of the administered dose (AD). The radioactivity found in the GI tract is likely to correspond to the unabsorbed dose and the fraction undergoing entero-hepatic recirculation whilst the radioactivity found in the liver is likely to represent the fraction undergoing metabolism. The other tissues examined had radioactivity levels corresponding to less than 1 % of the dose. About 13-24 % of the applied doses were found in carcasses. Since the percent of dose recovered in rat tissues and carcasses were similar in both sexes after single and multiple low (5 mg/kg bw) oral doses, it can be concluded that there is no accumulation of bixlozone and/or its metabolites in any tissue.

The radioactivity levels measured in tissues after the single high oral dose (500 mg/kg bw) showed that the extent of exposure increased with increased dose. The % of dose observed in each tissue at 500 mg/kg bw was consistent with that found at the lower dose (5 mg/kg), indicating an absence of selective accumulation of total radioactivity in any of the tissues upon administering a higher dose of [^{14}C-Phenyl]-bixlozone. Similar radioactivity levels were observed in tissues of both male and female rats, confirming no sex-difference in general tissue deposition.

At the approximate T_{max} of total radioactivity, the highest tissue to plasma ratios (Table B 6.1.1.13) were measured in the GI tract (up to 8 at the low dose, and 39 at the high dose), liver (up to 2 at the low and high doses), thyroid (up to 1.5 at the low dose, and 3.7 at the high dose), white fat (up to 0.7 at the low dose, and 9.7 at the high dose), adrenals (up to 1 at the low dose, and 3 at the high dose), kidney (0.6 to 1.4 at the low and high doses), lung (up to 1 at the low and high doses), ovaries and uteri (up to 0.5 at the low dose and at 2.7 at

high dose), heart (up to 0.7 at the low and high doses), spleen (up to 0.6 at the low and high doses), skin (<0.3 at the low dose and up to 2 at the high dose), brain (≤1 at the low and high doses), muscles (<0.2 at the low dose and up to 1 at the high dose), bone (<0.2 at the low dose and <0.4 at the high dose) and testes (<0.1 at the low dose and <0.4 at the high dose).

Mean concentrations (µg Eq/g) of total radioactivity in tissues, % radioactivity in tissues and tissue to plasma ratios of total radioactivity in male and female rats following single or multiple oral doses of [^{14}C-Phenyl]-bixlozone are summarised in the tables below.

Table B 6.1.1.11: Mean concentrations (µgEq/g) of total radioactivity in tissues and carcasses of male and female rats following administration of single (low and high) and multiple (low) oral dose with [^{14}C-Phenyl]-bixlozone (at T_{max})

Sample	Mean concentrations (µgEq/g) in tissue and carcass					
	Single oral Low dose Males	Single oral Low dose Females	Single oral High dose Males	Single oral High dose Females	Repeated oral Low dose Males	Repeated oral Low dose Females
Whole Blood	2.05	3.47	75.88	57.70	3.56	2.93
Plasma	4.27	6.82	139.31	99.54	7.21	6.03
Liver	8.26	11.67	222.46	210.80	10.29	9.22
Brain	0.45	1.56	56.59	65.33	0.69	1.09
Heart	0.98	2.09	67.66	66.03	1.63	1.65
Lungs	2.05	5.30	79.33	84.03	4.15	3.15
Fat*	1.68	4.73	655.00	970.84	2.41	2.54
GI tract	29.68	27.35	3447.40	3470.88	37.72	29.13
Kidneys	2.73	6.10	122.57	139.44	5.12	4.14
Spleen	0.99	1.80	48.94	56.14	1.50	2.08
Skin*	0.78	1.48	106.67	189.98	1.21	1.18
Adrenals	1.92	5.20	247.70	311.46	3.41	5.61
Testes	0.39	NA	51.52	NA	0.65	NA
Ovaries + Uteri	NA	2.63	NA	290.13	NA	3.16
Bone*	0.56	0.83	43.59	43.77	0.68	0.73
Thyroid	4.87	7.13	565.54	145.67	3.94	1.38
Muscles*	0.41	0.96	48.56	98.21	0.70	0.79
Carcass	0.66	1.26	99.01	105.64	0.96	0.96

* Only representative amount of tissue was collected (not whole tissue)

Table B 6.1.1.12: Percent radioactivity (as % of AD) in tissues and carcasses of male and female rats following administration of single (low and high) and multiple (low) oral dose with [^{14}C-Phenyl]-bixlozone (at T_{max})

Sample	Percent of dose recovered in rat tissues and carcasses (%)					
	Single oral Low dose Males	Single oral Low dose Females	Single oral High dose Males	Single oral High dose Females	Repeated oral Low dose Males	Repeated oral Low dose Females
Whole blood	1.04	1.81	0.38	0.31	1.39	1.22
Liver	6.63	8.04	1.69	1.48	7.33	6.45
Brain	0.07	0.26	0.09	0.11	0.08	0.17
Heart	0.09	0.17	0.06	0.05	0.13	0.12
Lungs	0.35	0.68	0.09	0.09	0.53	0.46
Fat*	0.05	0.13	0.27	0.30	0.05	0.12
GI tract	64.72	50.36	63.24	61.57	56.27	50.25
Kidneys	0.52	1.03	0.23	0.24	0.92	0.73
Spleen	0.06	0.08	0.02	0.02	0.05	0.10
Skin*	0.02	0.03	0.04	0.05	0.02	0.04
Adrenals	0.01	0.03	0.01	0.02	0.02	0.06
Testes	0.09	NA	0.12	NA	0.11	NA
Ovaries + uteri	NA	0.17	NA	0.23	NA	0.36
Bone*	0.06	0.08	0.05	0.05	0.06	0.09
Thyroid	0.04	0.06	0.05	0.01	0.03	0.01
Muscles*	0.04	0.09	0.04	0.06	0.07	0.04
Carcass	12.89	23.82	18.87	19.87	18.77	18.76
Total	**86.66**	**86.85**	**85.26**	**84.46**	**85.82**	**78.98**

* Only representative amount of tissue was collected (not whole tissue)

Table B 6.1.1.13: Mean tissue or carcasses to plasma concentration ratios of male and female rats following administration of single (low and high) and multiple (low) oral dose with [^{14}C-Phenyl]-bixlozone (at T_{max})

Sample	Mean concentration ratios of tissues and carcasses to plasma					
	Single oral Low dose Males	Single oral Low dose Females	Single oral High dose Males	Single oral High dose Females	Repeated oral Low dose Males	Repeated oral Low dose Females
Whole blood	0.48	0.51	0.54	0.58	0.50	0.49
Plasma	1.00	1.00	1.00	1.00	1.00	1.00
Liver	2.03	1.78	1.58	2.19	1.42	1.52
Brain	0.11	0.23	0.38	0.66	0.09	0.18
Heart	0.23	0.31	0.46	0.66	0.23	0.27
Lungs	0.50	0.87	0.56	0.87	0.57	0.52
White fat	0.38	0.69	4.52	9.69	0.33	0.44
GI tract	7.53	4.17	26.97	38.96	5.44	4.95
Kidneys	0.61	0.90	0.87	1.37	0.74	0.69
Spleen	0.26	0.28	0.34	0.58	0.21	0.34
Skin	0.18	0.22	0.76	2.00	0.17	0.20
Adrenals	0.46	0.78	1.65	3.00	0.46	0.92
Testes	0.09	NA	0.35	NA	0.09	NA
Ovaries + uteri	NA	0.39	NA	2.68	NA	0.54
Bone	0.14	0.12	0.30	0.44	0.09	0.12
Thyroid	1.41	1.15	3.67	1.37	0.51	0.23
Muscles	0.10	0.14	0.33	0.91	0.10	0.13
Carcass	0.16	0.19	0.67	1.06	0.13	0.16

Conclusion

The tissue distribution of bixlozone and/or its metabolites was determined at T_{max} in male and female Sprague-Dawley rats following single (low and high) or multiple (low) oral (gavage) doses of [^{14}C-Phenyl]-bixlozone in a GLP and OECD test guideline-compliant study.

In all dose groups, blood / plasma ratios of radioactivity did not indicate preferential partitioning into the red blood cells. A possible saturation of absorption was observed at the high dose (500 mg/kg bw) since the mean radioactive concentrations found in plasma and blood, compared to those recorded after a single oral low dose (5 mg/ kg bw), was not proportional to the increase in dose.

The data show that the overall distribution of [^{14}C-Phenyl]-bixlozone was rapid and extensive following oral dosing, independently of the dose level or frequency. No significant differences in the levels of radioactivity in tissues were found between single or multiple dose groups or between low and high dose groups, suggesting there is no accumulation of bixlozone and/or its metabolites in any specific tissue following high or repeated dosing.

Among all the tissues analysed at the T_{max}, the GI tract (≈ 50-65 % of dose), likely representing the unabsorbed dose and the fraction undergoing entero-hepatic recirculation, and the liver (≈ 1.5-8 % of dose), likely representing the fraction undergoing metabolism, had significant concentrations of radioactivity. The other tissues examined had approximately 1 % of the AD at both the low and high doses. About 13-24 % of the AD was found in the carcasses at T_{max}.

[redacted] (2017b)

Study 2 (bone marrow and blood distribution study)

This study was conducted in support of the *in vivo* micronucleus assay with the aim to demonstrate systemic and bone marrow exposure at the doses used in the *in vivo* rat bone marrow micronucleus assay described in detail in Section B.6.4.2)

Study # 5	Radioactivity concentration in plasma and bone marrow at T_{max} after oral administration of [^{14}C-Phenyl]F9600 to Sprague-Dawley rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2017f)
Date performed	November 2017
Test facility	[redacted]
Report reference	Study no. FMC-P7354
Guideline(s)	OECD 417 (2010)
Deviations from the guideline	None of significance
GLP	Yes
Test material	[^{14}C-Phenyl]-F9600 Batch CFQ43224; purity 99.8 %; specific activity 63 mCi/mmol (228.3 µCi/mg) F9600 Batch JB-F9600-201603004; purity 96.8 %
Study acceptable	Yes

Material and Methods

Four male Sprague-Dawley rats were administered a single oral high dose of [^{14}C-Phenyl]-bixlozone at 500 mg/kg bw by gavage, using the vehicle 0.5 % CMC and 5 % Tween 80. Bone marrow and blood for extraction of plasma were collected at 4 hours post-dose (T_{max}). The time point selected for necropsy and sample collection (4 hours post-dose) was based on the T_{max} determined in a previous pharmacokinetic study (please refer to Section B.6.1.1.2). Duplicate aliquots were taken and analysed for radioactivity content by LSC.

Results

All animals appeared to be in good health throughout the study and demonstrated no adverse effects from bixlozone administration.

The mean total radioactivity concentration was 153.20 µg Eq/g ± 32.46 in plasma and 49.73 µg Eq/g ± 11.94 in bone marrow. The mean bone marrow to plasma ratio was around 0.3, showing the bone marrow was well exposed to bixlozone and/or its metabolites following administration of an oral high dose at 500 mg/kg/bw.

Table B 6.1.1.4.5: [^{14}C-Phenyl]-bixlozone radioactivity concentrations in rat plasma and bone marrow at T_{max} following a single oral high dose at 500 mg/kg bw

Rat #	Total Radioactive Concentration			Mean Concentration (N=4 Rats)			
	DPM/g	nCi/g	µg Eq/g	DPM/g	nCi/g	µg Eq/g	SD
Plasma							
1M	131743	59.34	143.60	140543	63.31	153.20	32.46
2M	137522	61.95	149.90				
3M	181833	81.91	198.20				
4M	111073	50.03	121.07				
Bone marrow							
1M	43783	19.72	47.72	45627	20.55	49.73	11.94
2M	31445	14.16	34.28				
3M	57312	25.82	62.47				
4M	49969	22.51	54.47				

SD: Standard deviation
DPM: Disintegration per minute
µCi: Micro curie

Conclusion

The mean total concentration of [^{14}C-Phenyl]-bixlozone and/or its metabolites found in male rats at the T_{max} following a single oral dose of 500 mg/kg bw were 153.20 and 49.73 µg Eq/g in plasma and bone marrow, respectively; the mean bone marrow to plasma ratio was ≈ 0.3. Significant levels of total radioactivity were detected in bone marrow at the T_{max}, providing proof of systemic and bone marrow exposure at the doses used in the *in vivo* rat bone marrow micronucleus assay (Section B.6.4.2).

B.6.1.1.5. *Excretion and metabolism studies with [^{14}C-Phenyl]-bixlozone*

The excretion routes and metabolism of [^{14}C-Phenyl]-bixlozone were investigated in Sprague-Dawley (SD) rats following administration of single or repeated low doses at 5 mg/kg bw or a single high dose at 500 or 1000 mg/kg bw.

Study # 6	Excretion routes and metabolism of [^{14}C-phenyl]F9600 in male and female Sprague-Dawley rats following single or multiple oral doses
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2018b)
Date performed	July 2015 – March 2017
Test facility	[redacted]
Report reference	Study no. FMC-P3887
Guideline(s)	OECD 417 (2010)
Deviations from the guideline	Some deviations from the protocol occurred during the study: 1. For group 3M, only 3 animals were analysed to determine the mass balance and obtain metabolite profiles because one of the 4 animals were sacrificed 2 days after receiving the dose at 1000 mg/kg bw due to unexpected clinical signs. 2. For group 3M some of the faecal samples were limited in weight thus they have been homogenised in 10 mL of water instead of the 1 to 5 volumes of water specified in the protocol. 3. The group 4F was administered 500 mg/kg bw instead of 1000 mg/kg bw due to unexpected clinical observations in group 3M; the adjustment was necessary to avoid or minimise discomfort, distress or pain to animals. Overall HSE considers that the deviations listed above had no major effects on the outcome of the study results and should not have a significant impact on the validity of the study.
GLP	Yes
Test material	[^{14}C-Phenyl]-F9600 Batch 77874-3-19; purity 100 %; specific activity 56 mCi/mmol (202.9 µCi/mg) F9600 Batch PL 14-0163; purity 99.8 % (by HPLC)
Study acceptable	Yes

Material and Methods

[^{14}C-Phenyl]-bixlozone (200 µCi/kg) was administered orally by gavage to male and female Sprague-Dawley (SD) rats (n = 4 animals/sex) as a single or repeated (14 days) low dose at 5 mg/kg bw or as a single high dose at 500 mg/kg bw for females and 1000 mg/kg bw for males. The animal groups receiving repeated low oral doses were administered non-radiolabelled bixlozone from day 1 to day 13 followed by administration of [^{14}C-Phenyl]-bixlozone on day 14. The single high dose administered to females was reduced to 500 mg/kg bw following the observation of slight distress in animals from the male group treated at 1000 mg/kg bw.

Table B 6.1.1.14: Dosing design for [^{14}C-Phenyl]-bixlozone mass balance study in rats

Group #	Sex	# of rats	Dose route & regimen		Target dose concentration and volume				
			Route	Regimen*	µCi/kg	mg/kg bw	mg/mL	µCi/mL	mL/kg
1M	Male	4	PO	Day 1	200	5	0.5	20	10
2F	Female	4	PO	Day 1	200	5	0.5	20	10
3M	Male	4	PO	Day 1	200	1000	100	20	10
4F**	Female	4	PO	Day 1	200	500	50	20	10
5M	Male	4	PO	Day 1 to Day 14	200	5	0.5	20	10
6F	Female	4	PO	Day 1 to Day 14	200	5	0.5	20	10

**Dose was reduced to 500 mg/kg bw due to the observation of slight distress in one of the rats of group 3M (sacrificed at 24 hours post-dose).

Urine and faeces were collected for 7 days post-dose and selected tissues were collected following sacrifice at the end of the study (168 hours post-dose) from all dose groups (n = 4 animals/sex). Expired [^{14}C]CO_2 was trapped up to 48 hours post-dose (n = 2 animals/sex) following the single low dose at 5 mg/kg bw. Following sample processing the radioactivity concentrations were assessed in urine, faeces, tissues, carcass, cage wash, and expired [^{14}C]CO_2 by LSC or combination of combustion and LSC analysis to determine mass balance.

The metabolite profiles and identification of metabolites (≥ 5 % of radioactivity or as low as achievable) in pooled urine and faeces up to 72 hours post-dose were achieved using HPLC/β-RAM and LC/MS. The detection of the metabolite structures was optimised by carrying out enzymatic hydrolysis of the pooled urine samples using Glusulase® at 37°C for 24 hours before sample processing and analysis using HPLC/β-RAM and LC/MS. 4-Acetamidophenyl β-*D*-glucuronide sodium salt 20 µM (ADGSS) was used as a positive control.

Results

Clinical signs

The males from the group treated with 1000 mg/kg bw (group 3M) displayed a slight distress following treatment; one male from this group was sacrificed at 24 hours post-dose. Consequently, the high dose subsequently administered to females was adjusted to 500 mg/kg bw to avoid or minimise discomfort, distress or pain to animals. Regarding the repeated-dose groups (5 mg/kg bw) it was noted the males did not gain more than 5 % body weight by day 14 whilst females lost on average 8 % of their body weight by day 14.

Excretion / Mass Balance (Table B 6.1.1.15)

In both male and female rats, total radioactivity recovered in expired air (48 hours post-dose) was minimal, constituting only < 0.02 % of the AD. The total radioactivity found in selected tissues and carcasses by the end of the study (168 hours post-dose) was also low in all groups (mean recovery ≤ 1 % of the AD), suggesting that the majority of [^{14}C-Phenyl]-bixlozone and its related components were eliminated via urine and faeces. There were no significant differences in recovered radioactivity in tissues/carcass between the single and multiple oral dose groups, suggesting that bixlozone and its related compounds have no tendency to accumulate in tissues.

Radioactivity was rapidly excreted in urine and faeces following the single oral low and high doses, with a mean total recovery of radioactivity > 90 % within 168 hours post-dose for both sexes. The data also indicated that the main excretion route was via urine (64 % and 88 % of AD in males and females at 168 hr) and to a lesser extent via faeces (27 % and 11 % of AD in males and females at 168 hr) in both sexes, with a higher urinary excretion found in female rats compared to male rats, indicating high systemic exposure.

Following single or repeated low and/or high oral doses, radioactivity was mainly excreted via urine in rats indicating that the majority of dosed [^{14}C-Phenyl]-bixlozone was rapidly absorbed through the GI track, metabolised, and excreted in the forms of polar metabolites and/or unchanged parent compound through kidney and recovered in urine. Slight sex differences were observed in urinary excretion rates at both low and high doses; female rats had higher absorption and/or metabolism than male rats at similar dose levels and conditions. The radioactivity (10% to 30%) recovered in faeces could be from biliary excretion and/or unabsorbed dose.

The rate of elimination was high following the single or repeated oral low dose (5 mg/kg bw), with a mean total recovery of administered radioactivity > 70-89 % at 24 hours post-dose in both sexes. A slower elimination rate was noted at the high oral dose, with mean total recovery of radioactivity of ≈ 32-34 %, 69-72 %, and ≈ 88 % in the 24, 48, and 72 hour post-dose for both sexes (all excreta combined).

Overall, the majority of the radioactivity (> 90 %) was recovered in urine (64-88 % of AD) and faeces (11-27 % of AD) within 7 days. The percent excretion of the total administered dose was found to be higher in urine of female rats than those in males, regardless of dosing regimen. This suggests a higher systemic exposure in female rats. The results also indicate that bixlozone and/or its metabolites have no tendency to accumulate in tissues. Less than 0.02 % of the AD was eliminated in expired air and < 1 % of the AD was found in tissues.

In the absence of bile cannulation experiments following oral exposure, the radioactivity recovered in the faeces should be excluded for the purposes of determining the oral absorption value as it could represent unabsorbed material. Therefore, summing up the radioactivity in urine and tissues at the low dose of 5 mg/kg bw [^{14}C-Phenyl]-bixlozone (a dose relevant to reference value derivation), oral absorption appears to be **65 %** in males and 88 % in females. These values are consistent with the systemic bioavailability values calculated in the study by [redacted], 2016 for both sexes (Section B.6.1.1.2).

The mean (± SD) cumulative percent excretion of radioactivity in urine (including cage wash), faeces, expired CO_2, tissues, remaining carcasses, and total recovery as percent of dose in male and female rats are summarised in the table below:

Table B 6.1.1.15: Mean (± SD) cumulative percent excretion of radioactivity in urine, faeces and expired air of male and female SD rats following administration of single (low or high) and multiple oral doses of [^{14}C-Phenyl]-bixlozone (mean ± SD; n=4*)

Group #	Dose (mg/kg bw)	Matrix/Route	% Dose		
			0-24 hr	0-48 hr	0-168 hr
1M (Male)	5 (single)	Urine#	54.06 ± 3.75	60.75 ± 3.55	64.17 ± 3.03
		Faeces	16.41 ± 3.37	22.58 ± 3.41	26.58 ± 2.81
		[^{14}C]CO_2	0.01 ± 0.00	0.02 ± 0.00	NA
		Tissues/Carcass	NA	NA	1.10 ± 0.64
		Total**	70.47 ± 3.37	83.34 ± 3.79	91.86 ± 0.95
2F (Female)	5 (single)	Urine#	80.42 ± 7.46	85.79 ± 6.16	87.52 ± 5.56
		Faeces	8.60 ± 3.49	10.79 ± 4.46	11.44 ± 4.71
		[^{14}C]CO_2	0.01 ± 0.00	0.01 ± 0.00	NA
		Tissues/Carcass	NA	NA	0.32 ± 0.21
		Total	89.02 ± 4.13	96.59 ± 1.77	99.29 ± 0.78
3M* (Male)	1000 (single)	Urine#	29.39 ± 5.96	59.90 ± 2.27	63.81 ± 9.29
		Faeces	4.11 ± 3.02	12.56 ± 10.96	21.06 ± 5.56
		Tissues/Carcass	NA	NA	0.60 ± 0.25
		Total	33.50 ± 8.77	72.47 ± 8.75	95.86 ± 4.77
4F (Female)	500 (single)	Urine#	31.04 ± 6.15	64.70 ± 10.67	81.71 ± 2.77
		Faeces	0.80 ± 0.90	4.57 ± 2.84	10.41 ± 3.40
		Tissues/Carcass	NA	NA	0.09 ± 0.07
		Total	31.85 ± 5.54	69.27 ± 12.54	92.22 ± 4.12
5M (Male)	5 (repeated)	Urine#	56.14 ± 8.71	59.77 ± 5.71	61.84 ± 3.83
		Faeces	19.82 ± 2.60	27.85 ± 2.26	33.77 ± 0.35
		Tissues/Carcass	NA	NA	0.26 ± 0.13
		Total	75.96 ± 11.12	87.62 ± 7.65	95.87 ± 3.86
6F (Female)	5 (repeated)	Urine#	71.87 ± 7.73	77.37 ± 5.76	78.99 ± 5.07
		Faeces	10.51 ± 3.34	12.77 ± 4.21	13.38 ± 4.52
		Tissues/Carcass	NA	NA	0.39 ± 0.18
		Total	82.38 ± 4.54	90.14 ± 1.77	92.76 ± 0.86

Including cage wash; NA: not available

* n = 3 for Group 3M (due to observation of slight distress, rat M9 was sacrificed after 24 hr post-dose)

** Total of 168 hr included radioactivity in urine, faeces, CO_2, tissues and carcass

Metabolism (Table B 6.1.1.16)

Chromatographic analysis of urine and faeces samples showed that bixlozone was extensively metabolised in rats following a single low, high or repeated low oral gavage doses of [^{14}C-Phenyl]-bixlozone. Unchanged parent compound (bixlozone) was only detected at low levels (<1 % of the AD) in male rat urine after a single 1000 mg/kg bw oral dose. More than 40 different metabolites were observed in urine samples, with fewer metabolites retrieved in faeces samples. The metabolite profiles drawn from the urine and faeces samples were overall qualitatively similar between male and female rats.

In pooled urine samples, the major metabolites (defined as present at levels ≥ 10 % the AD in both sexes) were 2,4-dichlorohippuric acid and 5-keto-hydrate-bixlozone. In males, metabolites identified at > 5 % of the AD were 5-OH-bixlozone glucuronide (group 3M), bixlozone-cysteine derivative (group 1M & 5M) and dimethyl malonamide bixlozone (group 1M & 5M). In females, metabolites present at levels > 5 % of the AD were 5-OH-bixlozone glucuronide (group 2F), bixlozone -cysteine derivative (all females groups) and dimethyl malonamide bixlozone (group 4F). Minor urinary metabolites, representing approximately 2-5 % of the AD, included Mw467a glucuronide (RP3), Mw277a (RP11), Mw449 glucuronide (RP16), Mw437 glucuronide (RP19), Mw453b glucuronide (RP20), 3-OH-propanamide glucuronide (RP21), dimethyl malonamide-bixlozone glucuronide (RP24), Mw341 sulfate (RP30), and Mw321 (RP39). Lastly, several metabolites were detected at very low levels (< 2 % of the AD) and were structurally identified when possible. The major metabolites present in urine samples were not present in faeces, however some minor metabolites were common to both matrices (5-OH- bixlozone glucuronide and dimethyl malonamide- bixlozone).

In pooled faeces samples in males, the metabolite present at levels ≥ 10% of the AD was 3-OH-propanamide bixlozone (group 1M & 5M); the metabolite 5-OH-bixlozone was present at levels close to 10 % (group 3M &

5M) and dimethyl malonamide-bixlozone at ≈ 5 % of the AD (group 1M & 5M). In females the only faecal metabolite seen at > 5 % of the AD was 3-OH-propanamide bixlozone (group 2F & 6F). Minor metabolites (accounting for 1 – 5 % of the AD) detected in faeces included OH-isobutyramide-bixlozone (RP31) and 5-OH-bixlozone glucuronide (RP27). It was noted that 5-OH-bixlozone detected in faeces was excreted in urine as a glucuronide conjugate.

Based on the metabolites identified in urine and faeces, it is proposed that the dimethylisoxazolidin-3-one ring moiety of bixlozone was the most susceptible site of metabolism in rats. A combination of various metabolic reactions (oxidation, ring-scission, decarboxylation) on this moiety appear to lead to various metabolites including oxidative ring-opened analogues, ring-cleaved analogues; one-ring structure metabolites are also produced following cleavage of the two-ring structure. The phase I metabolites, produced by various metabolic pathways, are subsequently conjugated and excreted as glucuronides in rat urine.

The metabolites characterised and their percent distribution in urine and faeces of male and female rats are summarised in the table below:

Table B 6.1.1.16: Percentages of radioactive components found at levels > 5 % of the administered dose in urine (up to 72 hr) and faeces (0-48 hr) after a single low or high oral dose, and repeated-dose of [^{14}C-Phenyl]-bixlozone to male and female SD rats

Metabolite #	% administered dose											
	Single oral low dose (5 mg/kg bw)				Single oral high dose (1000 / 500 mg/kg bw, M/F)				Repeated oral low dose (5 mg/kg bw)			
	Urine		Faeces		Urine		Faeces		Urine		Faeces	
	1M	2F	1M	2F	3M	4F	3M	4F	5M	6F	5M	6F
2,4-Dichlorohippuric Acid (RP15*)	**11.7**	**14.5**	-	-	9.8	15.0	-	-	6.8	12.15	-	-
5-OH-bixlozone glucuronide (RP27)#	2.3	6.0	-	-	5.4	4.0	-	-	1.3	1.0	-	-
5-keto-hydrate-bixlozone (RP33, Mw341)	**17.6**	**24.3**	-	-	**13.6**	**24.8**	-	-	**34.6**	**29.7**	-	-
Bixlozone-cysteine derivative (RP34, Mw364)	6.6	10.1	-	-	2.2	5.1	-	-	6.2	20.1	-	-
3-OH-propanamide bixlozone (RP35)	-	-	12.9	5.3	0.1	-	0.6	4.1			12.6	6.1
Dimethyl malonamide bixlozone (RP38)	5.8	4.2	5.1	2.2	2.4	6.7	-	0.5	6.5	4.0	5.45	1.6
5-OH-bixlozone (RP41)	-	-	3.3	2.9	0.4	0.4	9.7	-	-	-	9.0	2.6
Bixlozone (RP46)	-	-	-	-	0.6	-	1.7	-	-	-	-	-
Others**	14.8	21.0	1.25	0.5	26.9	14.8	0.7	0	4.2	6.4	0.8	2.55
Total % of the administered dose	**58.85**	**80.2**	**22.6**	**10.8**	**61.4**	**70.85**	**12.75**	**4.6**	**59.7**	**73.4**	**27.85**	**12.8**

M: males; F: females

Confirmed by enzyme hydrolysis of urinary metabolites.

* The metabolites identified in this study were expressed as RP1 to RP48 for urine and faeces metabolites based on retention times of each radioactive peak in each matrix.

**The sum of minor radioactive metabolites, each representing < 5 % of administered dose, present in urine or faeces.

Conclusion

This OECD guideline and GLP-compliant study showed that orally administered [^{14}C-Phenyl]-bixlozone and/or its metabolites were rapidly excreted via urine and faeces in Sprague-Dawley rats. Neither dose level, frequency of administration, or sex were significant modulating factors in the pattern of excretion. Urinary excretion was the major route of elimination, accounting for about 62 – 64 % of the AD in males and 79 – 88 %

of the AD in females. The total radioactivity found in selected tissues and carcasses at 168 hours post-dose was low in all groups, suggesting that bixlozone and/or its metabolites have no tendency to accumulate in tissues.

In the absence of bile cannulation experiments following oral exposure, the oral absorption appears to be **65 %** in males and 88 % in females by summing up the radioactivity in urine and tissues at the low dose of 5 mg/kg bw [^{14}C-Phenyl]-bixlozone (a dose relevant to reference value derivation) and excluding the radioactivity recovered in the faeces as it could represent unabsorbed material. These values are consistent with the systemic bioavailability values calculated in the study by [redacted], 2016 for both sexes (Section B.6.1.1.2).

Bixlozone was extensively metabolised in rats; hydroxylation leading to the formation of 5-OH-bixlozone and its derivatives appear to be a major metabolic pathway. Other routes of metabolism included a combination of oxidation, decarboxylation and deamination followed by conjugation of oxidative derivatives. Further, the opening of the dimethylisoxazolidin-3-one ring and the cleavage of the 2-ring structures lead to a large number of two-ring and single ring metabolites to complete the metabolisation of bixlozone. The phenyl ring is well conserved through the metabolic process.

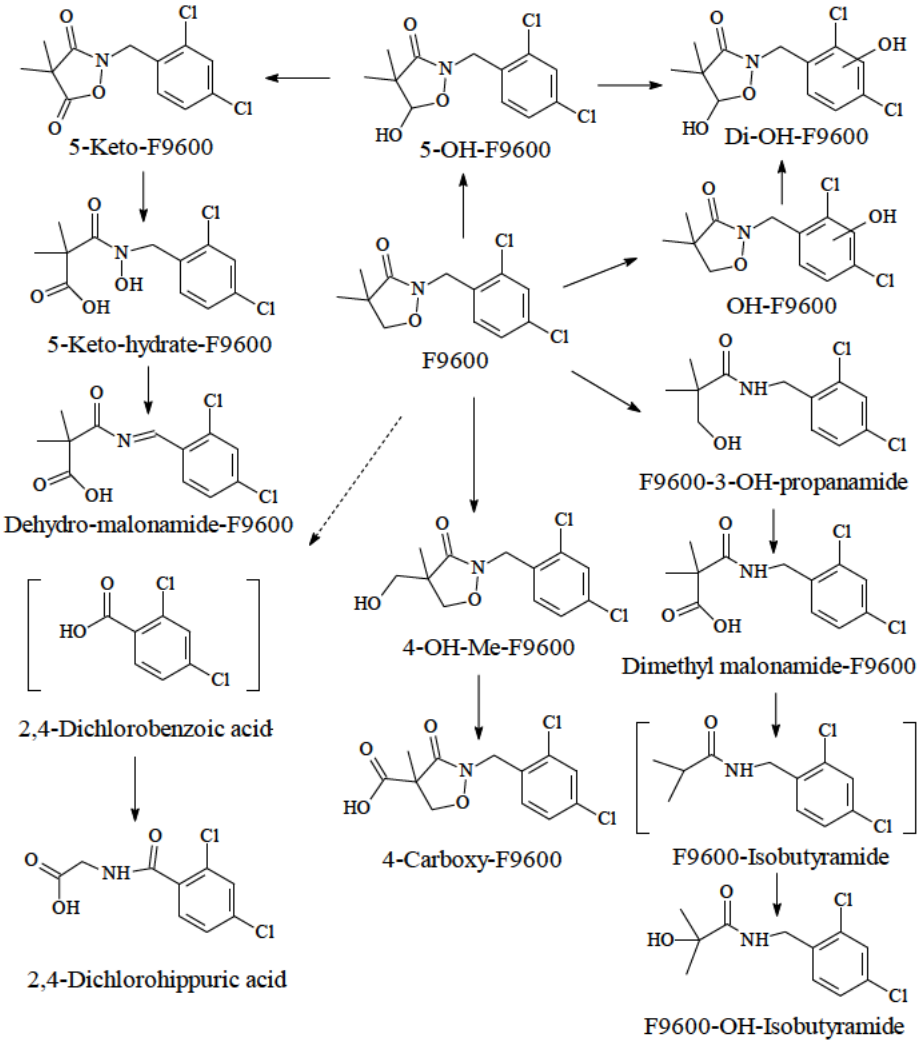
There were no significant differences observed in metabolite profiles between both sexes. The major metabolites identified in both sexes but exclusively in urine (and not in faeces) were 2,4-dichlorohippuric acid and 5-keto-hydrate-bixlozone, whilst the major metabolite identified exclusively in faeces was 3-OH-propanamide-bixlozone.

The proposed metabolic pathways for [^{14}C-Phenyl]-bixlozone in rats are shown in the figure below:

Several of the metabolites were present as conjugates in urine.

Minor metabolites and metabolites that were not fully characterised are not shown in the pathway scheme.

Figure B 6.1.1.1: Proposed metabolic pathways of [^{14}C-Phenyl]-bixlozone in male and female rats



B.6.1.1.6. *ADME studies conducted with [^{14}C-Carbonyl]-bixlozone*

Two excretion and metabolism oral studies conducted with [^{14}C-Carbonyl]-bixlozone are available, a pilot and a main study.

Study 1 –pilot study

A pilot study was conducted in male Sprague-Dawley rats to investigate the excretion routes, mass balance and metabolite profiles in urine and faeces of [^{14}C-Carbonyl]-bixlozone following a single low oral dose (gavage).

Study # 7	Metabolism of [^{14}C-carbonyl]F9600 in male and female Sprague-Dawley rats – pilot study
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2018a)
Date performed	July 2014
Test facility	[redacted]
Report reference	Study no. FMC-R3449
Guideline(s)	OECD 417 (2010)
Deviations from the guideline	N/A
GLP	Not to GLP – pilot study
Test material	[^{14}C-Carbonyl]-F9600 Batch CFQ42018; purity 99.9 %; specific activity 59 mCi/mmol (213.8 µCi/mg) Non-radiolabelled F9600 Batch G3773:62 (PL14-0163); purity 99.5 %
Study acceptable	Yes, but as supplementary information only since this is a pilot study

Material and Methods

Two male and two female Sprague-Dawley rats were administered a single oral gavage dose of [^{14}C-Carbonyl]-bixlozone (210 µCi/kg) at 5 mg/kg bw. After dosing, the animals were placed in individual metabolism cages with airtight enclosure under controlled air-flow and air washing systems to collect expired [^{14}C]CO_2, urine, and faeces. Urine and faeces were collected for up to 7 days post-dose whist expired air was collected for up to 72 hours post-dose. Selected tissues and blood were collected after sacrifice at 168 hours for total radioactivity determination.

Radioactivity concentrations in urine, faeces, cage wash, expired CO_2, tissues, and remaining carcasses were determined by LSC or combination of combustion and LSC analysis. The metabolite profiles and characterisation of the metabolites found in pooled urine and faeces were obtained by HPLC/UV/β-RAM and LC/MS/MS and/or comparison with the following synthetic reference standards: bixlozone, 5-keto-hydrate-bixlozone, bixlozone-3-OH-propanamide, bixlozone -dimethyl malonamide and 5-OH-bixlozone.

The collected raw data were used to determine the excretory balance of radioactivity (total amount of radioactivity in urine, faeces, and cage washes) and the total recoveries (expressed as % of AD). Concentration values below the limit of quantification were expressed as BQL (below the limit of quantification).

Results

Mortality / morbidity / body weights

Not reported.

Radioactivity concentrations in urine, faeces, expired air (CO_2), cage wash, tissues and carcasses

In male rats, the mean total recovery of radioactivity in excreta was 91 % of the AD, which is acceptable. The major excretion route was via the urine, accounting for 67 % of the AD within 168 hours post-dose. The radioactivity recovered in faeces accounted for 22 % of the AD. Less than 2 % of the AD was recovered via expired CO_2 by 72 hour post-dose whilst a negligible amount (0.6 % of the AD) was recovered in selected tissues and carcasses at 168 hours post-dose. Furthermore, it was observed that excretion was rapid: a total of 84 % of the dose was recovered within the first 48 hours post-dose, with 62 % of the dose found in the urine and 20 % recovered in faeces.

In female rats, the mean total recovery of radioactivity in excreta was 94 % of the AD, which is acceptable. Urinary excretion was higher in female rats (74 %) compared to male rats (67 %) whilst faecal excretion was lower in females (17 %) compared to male rats (22 %).

Metabolite profile in urine and faeces ***(Error! Reference source not found.)***

Unchanged bixlozone was not detected in urine, indicating the compound was extensively metabolised following oral dosing to rats. The major metabolites (> 10 % of the AD in urine in both sexes) found in urine only were carbamic acid (16 % in males; 22 % in females) and 5-keto-hydrate bixlozone (26 % in males, 23 % in females).

Table B 6.1.1.17: Distribution of bixlozone metabolites characterized in excreta (0-48 hr) of male and female rats following a single oral dose of [^{14}C-Carbonyl]-bixlozone at 5 mg/kg bw

Metabolite	Urine				Faeces				Total (% Dose)			
	Male		Female		Male		Female		Male		Female	
	% ROI	% Dose	% ROI	% Dose	% ROI	% Dose	% ROI	% Dose	% ROI	% Dose	% ROI	% Dose
Carbamic acid (U1)	**25.82**	**16.07**	**32.42**	**21.97**	NA	NA	NA	NA	19.63	16.07	26.68	21.97
Mw465 (U2)	NA	NA	3.71	2.52	NA	NA	NA	NA	0.00	0.00	3.06	2.52
Mw341 (U3)	8.99	5.60	6.57	4.45	NA	NA	NA	NA	6.84	5.60	5.40	4.45
5-keto-hydrate-bixlozone (U4, Mw305)	**42.52**	**26.47**	**33.72**	**22.85**	NA	NA	NA	NA	32.33	26.47	27.75	22.85
Bixlozone-3-OH-propanamide (U5/F1, Mw275)	2.98	1.86	6.43	4.36	57.18	11.21	60.52	8.82	15.97	13.07	16.00	13.17
Bixlozone-dimethyl malonamide (U6/F2, Mw289a)	9.78	6.09	12.42	8.42	32.62	6.40	25.92	3.78	15.25	12.49	14.81	12.20
5-OH-bixlozone (U7/F3, Mw289b)	2.66	1.66	1.46	0.99	5.24	1.03	NA	NA	3.28	2.69	1.20	0.99
Total identified (% of dose)	92.74	57.74	96.73	65.56	95.05	18.64	86.44	12.59	93.30	76.38	94.91	78.15

* present as a free form as well as conjugates
** present as a glucuronide conjugate form (P11 or P14) in urine
ROI: Region of Interest

Conclusion

In a pilot study conducted in line with the principles of OECD 417, a single oral gavage dose of [^{14}C-Carbonyl]-bixlozone was administered at 5 mg/kg (100 µCi/kg) to male and female rats. Recovery was acceptable, with high urinary excretion (67-74 % of AD) and lower faecal elimination (17-22 % of AD). Less than 2 % of AD was found in expired air and 0.6 % of the AD was recovered in tissues. It was also found that bixlozone was rapidly and extensively metabolised. The major metabolites carbamic acid (16 % in males; 22 % in females) and 5-keto-hydrate bixlozone (26 % in males, 23 % in females) were found in urine only.

[redacted] (2018a)

Study 2 – main study

The excretion routes, mass balance and metabolism of [^{14}C-Carbonyl]-bixlozone were investigated in this main study in male and female Sprague-Dawley rats following administration of a single oral (gavage) dose at 5 mg/kg bw.

Study # 8	Excretion routes and metabolism of [^{14}C-carbonyl]F9600 in male and female Sprague-Dawley rats following a single oral dose
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2017c)
Date performed	August 2016 – September 2017
Test facility	[redacted]
Report reference	Study no. FMC-P4547
Guideline(s)	OECD 417 (2010)
Deviations from the guideline	None.
GLP	Yes. Minor shortcomings were highlighted during the study audit however the study is considered acceptable for regulatory purposes.
Test material	[^{14}C-Carbonyl]-F9600 Batch CFQ42476; purity 99.9 %; specific activity 59 mCi/mmol (213.8 µCi/mg) F9600 Batch PL G3773-17; purity 99.5 % (by HPLC)
Study acceptable	Yes

Material and Methods

The main study followed the pilot study protocol using 4 animals / sex. The detection of the metabolite structures in pooled urine and faeces was further optimised by carrying out enzymatic hydrolysis using Glusulase® at 37°C for 24 hours before proceeding to HPLC/β-RAM and LC/MS.

Results

Excretion and mass balance (Table B 6.1.3.2)

Following a single oral low dose of [^{14}C-carbonyl]-bixlozone at 5 mg/kg bw the radioactivity was rapidly and virtually completely excreted in urine and faeces in male and female rats by the end of the study. The mean total recovery of radioactivity was > 93 % of the AD for both sexes within 168 hours post-dose, which is considered acceptable; 88 % of the AD was already recovered within 48 hours post-dose from both male and female rats. Urinary excretion accounted for the majority of recovered radioactivity (62 – 75 % of the AD), followed by faecal excretion (16 – 34 % of the AD). The radioactivity recovered in expired air ([^{14}C]CO_2), in selected tissues and carcasses was low for all animals.

Table B 6.1.1.18: Mean (±SD) percent of radioactive dose recovered in urine, faeces, expired CO_2, tissues, and carcasses of male and female Sprague-Dawley rats following a single 5 mg/kg oral dose of [^{14}C-Carbonyl]-bixlozone

Group	Sex	Matrix	% Dose		
			0-24 hr (N = 4)	0-48 hr (N = 4)	0-168 hr (N = 4)
1	Male	Urine*	46.23 ± 5.19	57.09 ± 4.93	62.19 ± 4.38
		Faeces	22.61 ± 2.34	29.64 ± 2.65	34.23 ± 4.07
		[^{14}C]CO_2**	1.39 ± 0.23	1.57 ± 0.25	NA
		Tissues / Carcass	NA	NA	1.01 ± 0.19
		Total	**69.54 ± 3.54**	**87.51 ± 1.88**	**98.24 ± 1.28**
2	Female	Urine**	64.29 ± 11.79	72.28 ± 11.26	75.72 ± 10.34
		Faeces	11.81 ± 5.12	15.20 ± 6.99	16.40 ± 7.57
		[^{14}C]CO_2	1.39 ± 0.20	1.52 ± 0.16	NA
		Tissues / Carcass	NA	NA	0.48 ± 0.20
		Total	**76.79 ± 6.65**	**88.24 ± 4.61**	**93.39 ± 3.66**

*Urine including cage wash; **n=2 for [^{14}C]CO_2, 0-72 hr; NA: not available

Radioactivity concentrations in whole blood and plasma

The radioactivity concentrations in plasma aliquots collected at sacrifice (168 hours post-dose) were low (within 2-fold of the Limit of Detection (LOD) of 40 dpm). This is consistent with the results obtained after a single oral administration of [^{14}C-Phenyl]-bixlozone at 5 mg/kg bw (Section B.6.1.1.2).

*Metabolism (**Error! Reference source not found.**)*

Chromatographic analysis of pooled urine and faeces samples showed that bixlozone was extensively metabolised in rats following a single oral dose of [^{14}C-Carbonyl]-bixlozone at 5 mg/kg bw. Unchanged parent compound (bixlozone) was not detected in urine or faecal samples. More than 20 metabolites were observed in urine samples whilst fewer metabolites were identified in faeces. All of the metabolites seen in faeces were also present in urine samples, although some of them were present in urine as conjugates.

In pooled urine samples the major metabolites (≥ 10 % of the AD in both sexes) were carbamic acid (which was not identified using [^{14}C-Phenyl]-bixlozone) and 5-keto-hydrate-bixlozone; 5-OH-bixlozone glucuronide represented 5-10 % of the AD. The remaining metabolites were minor and each of these metabolites represented < 5 % of the AD.

In pooled faeces samples the major metabolite identified was 5-OH-bixlozone. Other prominent metabolites (between 5 and 10 % of AD) were bixlozone-3-OH-propanamide and bixlozone-dimethyl malonamide, whilst minor metabolites were carbamic acid and bixlozone-OH-isobutyramide.

Based on the metabolites identified in urine and faeces, it is proposed that the dimethylisoxazolidin-3-one ring moiety of bixlozone was the most susceptible site of metabolism in rats, with the phenyl ring remaining relatively well conserved. A combination of various metabolic reactions (oxidation, ring-scission, decarboxylation) on this moiety appear to lead to a large number of metabolites including oxidative ring-opened analogues and ring-cleaved analogues. The phase I metabolites, produced by various metabolic pathways, are subsequently conjugated as glucuronides in the urine.

The metabolites characterised and their percent distribution in urine and faeces of male and female rats are summarised in the table below:

1 Percentages of radioactive components (of the administered dose) found in urine and faeces after a single low oral dose of [^{14}C-Carbonyl]-bixlozone to male and female SD rats

Metabolite #	% administered dose					
	Urine		Faeces		Total (%)[a]	
	Males	Females	Males	Females	Males	Females
Carbamic acid (U1/F1)	**10.35**	**17.54**	1.28	4.66	11.63	22.2
bixlozone-OH-isobutyramide-glucuronide (U8, Mw437)	1.26	1.82	NA	NA	1.26	1.82
U9 (Mw453)	1.99	3.94*	NA	NA	1.99	3.94
bixlozone-3-OH-propanamide-glucuronide (U10, Mw451)	0.95	4.06*	NA	NA	0.95	4.06
5-OH- bixlozone-glucuronide (U11, Mw465)	5.01	6.24	NA	NA	5.01	6.24
bixlozone-dehydro malonamide (U12, Mw287a)	1.03	2.6	NA	NA	1.03	2.6
bixlozone-OH-isobutyramide (F2, Mw261)	NA	NA	1.56	2.03	1.56	2.03
5-Keto-hydrate-bixlozone (U14, Mw305)	**17.25**	**22.97**	NA	NA	17.25	22.97
U15 (Mw364)	0.99	0.88	NA	NA	0.99	0.88
bixlozone-3-OH-propanamide (F3, Mw275)	NA	NA	9.17	2.54	9.17	2.54
bixlozone-dimethyl malonamide (U16/F4, Mw289a)	3.89	3.81	7.54	3.29	13.77	9.95
U17 (Mw259)	2.94**	3.47	NA	NA	2.94	3.47
5-OH-bixlozone (U18/F5, Mw289b)	1.03**	0.7	10.08	2.68	11.11	3.37
4-COOH-Me-bixlozone (U19, Mw303)	3.70**	1.45	NA	NA	3.7	1.45
5-Keto-bixlozone (U20, Mw287b)	0.52	0.71	NA	NA	0.52	0.71
Total Identified (%)	**47.28**	**67.16**	**29.63**	**15.2**	**76.91**	**82.36**

NA: not available.

*Estimated due to co-eluting peaks.

**Estimated due to co-eluting peaks.

[a] In 0-48 hour period.

Conclusion

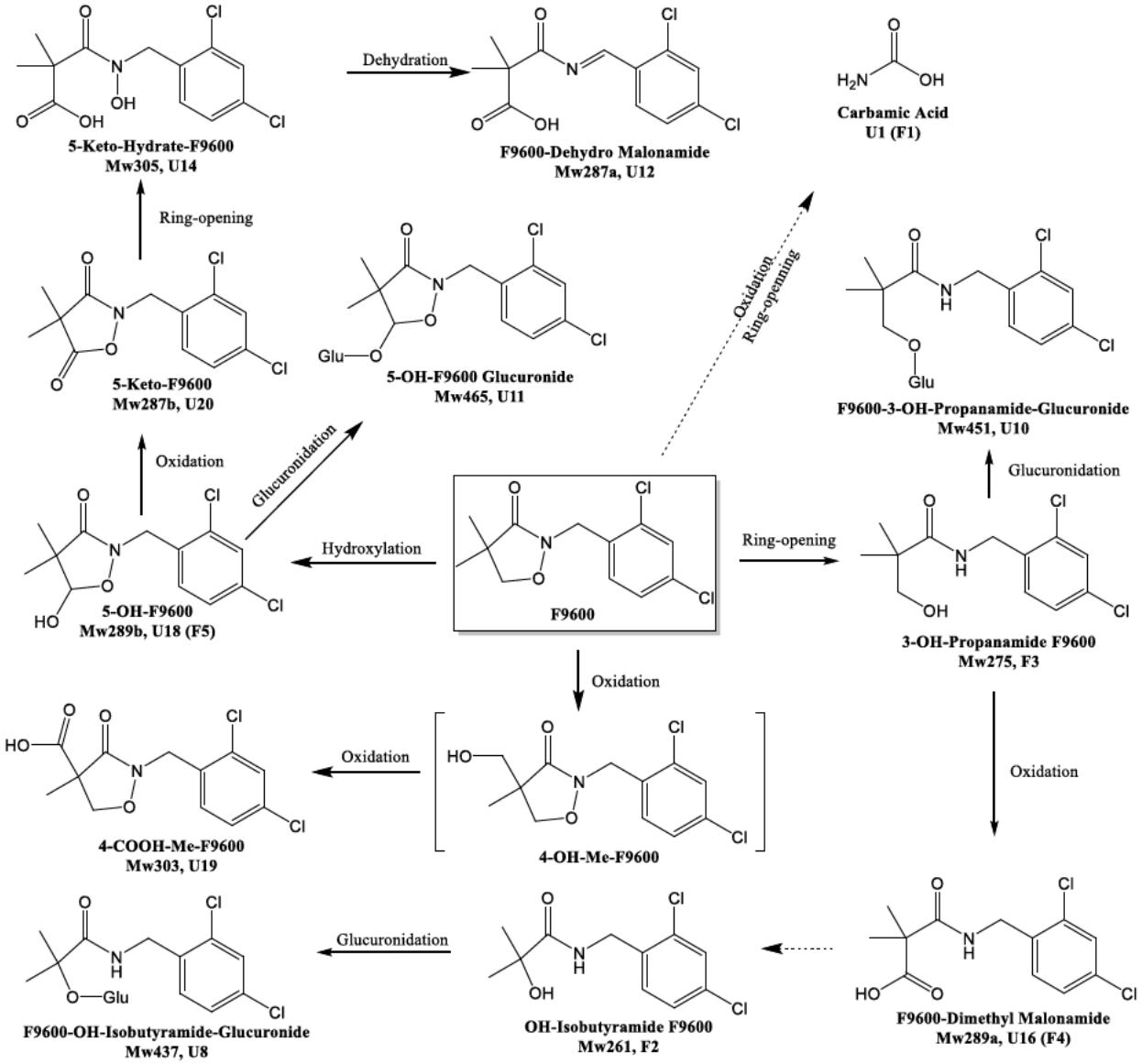
[^{14}C-Carbonyl]-bixlozone was rapidly excreted in urine and faeces of male and female Sprague-Dawley rats following administration of a single oral low dose of 5 mg/kg bw. The primary route of excretion was *via* urine, with a higher percentage of the dose found in females (76 % of the AD) compared to males (62 % of the AD). The secondary route of excretion was via the faeces (16-34 % of the AD); for both sexes a negligible percent of the AD (< 1.6 %) was recovered in exhaled air; there was also no apparent accumulation of bixlozone and/or its metabolites in tissues.

In the absence of bile cannulation experiments following oral exposure, the radioactivity recovered in the faeces should be excluded for the purposes of determining the oral absorption value as it could represent unabsorbed material. Therefore, summing up the radioactivity in urine and tissues at the low dose of 5 mg/kg bw [^{14}C-Carbonyl]-bixlozone (a dose relevant to reference value derivation), oral absorption appears to be **63 %** in males and 76 % in females. These values are similar to those obtained from a similar study conducted with bixlozone labelled in the phenyl ring (please refer to Section B6.1.1.2).

Bixlozone was extensively metabolised in rats, since unchanged bixlozone was not detected in urine or faecal samples. No significant sex differences were observed in the metabolite profiles. The major metabolites identified in urine in both sexes were carbamic acid and 5-keto-hydrate-bixlozone. Although carbamic acid was identified only in this study in which bixlozone labelled at the carbonyl group was administered, this metabolite must have also occurred when the phenyl label was used; however, it could not be detected due to the position of the label. Hydroxylation leading to the formation 5-OH-bixlozone and its derivatives appear to be a major metabolic pathway in rats. Other routes of metabolism included a combination of oxidation, decarboxylation and deamination followed by conjugation of oxidative derivatives.

[redacted] (2017c)

Figure B 6.1.1.2: Proposed metabolic pathways of bixlozone in male and female rats following a single 5 mg/kg oral dose of [^{14}C-Carbonyl]-bixlozone



Note: U7, U9, U15, and U17 were not included in the metabolic pathway, since each of these metabolites represented < 5% of the dose. U21 was observed only in hydrolysed urine samples, therefore, it is not included in this metabolic pathway. Please refer to **Error! Reference source not found.** for details.

B.6.1.2. Absorption, distribution, metabolism and excretion by other routes

Since > 20 % radioactivity was excreted via faeces, at least in male rats, following oral administration of bixlozone ([redacted] (2018b)), the excretion routes, mass balance and metabolism of [^{14}C-Phenyl]-bixlozone were investigated in bile duct cannulated male Sprague-Dawley rats following an IV dose at 3 mg/kg bw.

Study # 9	Mass Balance of [^{14}C-phenyl]-F9600 in male bile duct cannulated Sprague-Dawley rats following a single intravenous dose
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2017d) Amended in 2019 (Revision No. 1)
Date performed	July 2016 – April 2017
Test facility	[redacted]
Report reference	Study no. FMC-P5709
Guideline(s)	OECD 417 (2010)
Deviations from the guideline	None of significance
GLP	No. The study is claimed to be GLP compliant however the US Environmental Protection Agency (US EPA) was requested to conduct a GLP compliance inspection and an audit on this study. It was concluded that "The quality assurance does not comply with GLP and it seems that the responsible QA-employee was not sufficiently familiar with the type of investigation. Various measurement results have not been correctly reported. Measurement results reported in the summary do not correspond to values in tables. The report is amended to correct errors." Therefore, the study was concluded not to be GLP compliant. Following the outcome of the audit the report has been amended ([redacted] Report Amendment No. FMCR5709AM1) to correct the typographical errors and transcribing errors from electronic raw data. The report was submitted to HSE. Overall there was no impact on the study results or conclusions.
Test material	[^{14}C-Phenyl]-F9600 Batch 77874-3-19; purity 100 %; specific activity 56 mCi/mmol (202.9 µCi/mg) F9600 Batch G3773:17; purity 99.5 % (by HPLC)
Study acceptable	No – not relied upon. The study is not compliant with GLP. Also, as IV administration was used instead of oral administration, extrapolation of the biliary excretion data to the oral route does not seem appropriate as kinetics (and in particular biliary excretion) following IV administration are likely to be different from those following oral administration. Lastly, the study was performed in males only. Study presented for completeness only.

Material and Methods

Bile duct cannulated male Sprague-Dawley rats (n = 5) were administered a single IV dose of [^{14}C-Phenyl]-bixlozone at 3 mg/kg bw (150 µCi/kg). The bile was collected for up to 72 hours post-dose whilst urine and faeces were collected for up to 5 days after dosing. Cage wash was collected at the end of the study on day 5. The radioactivity in bile, urine, faeces, and cage wash was determined by LSC or combination of combustion and LSC analysis.

The metabolite profiles and identification of metabolites was conducted in bile and urine samples pooled up to 24 hours post-dose and using HPLC/β-RAM and LC/MS. Aliquots of 0-24 hour pooled urine and bile samples were also subjected to enzymatic hydrolysis to release the conjugated forms of metabolites (please refer to Section B.6.1.1.5 for more details).

Results

Excretion routes and mass balance ***(Error! Reference source not found.)***

The radioactivity was rapidly excreted in bile and urine, with greater than 90 % of the AD recovered within 24 hours post-dosing. The majority of the recovered radioactivity, about 52 % of the AD, was excreted in bile. Urine was also a major route of excretion, accounting for about 40 % of the AD. Minimal radioactivity, (approximately 1 % of the AD), was excreted through faeces, and is likely it was due to gastric secretion.

Overall the data indicate that the great majority of [^{14}C-Phenyl]-bixlozone and/or its metabolites are excreted mainly via the bile and urine within 24 hours after IV dosing. The excretion data are summarised in the table below:

Table B 6.1.2.1: Mean (± SD) cumulative percent excretion of radioactivity in bile, urine, faeces and total recovery as % of dose in bile duct cannulated male rats

Matrix	% administered dose			
	0-8 h (n=5)	0-24 hr (n=5)	0-72 hr (n=5)	0-120 hr (n=5)
Bile	49.17 ± 22.67	51.61 ± 23.60	52.11 ± 23.73	NA
Urine	27.15 ± 17.34	38.36 ± 22.33	39.73 ± 22.93	39.94 ± 22.98
Faeces	NA	1.07 ± 1.16	1.24 ± 1.42	1.29 ± 1.42
Total recovered*	76.32 ± 6.7	91.03 ± 1.50	93.08 ± 1.59	93.63 ± 1.89

* includes cage wash

NA: Not applicable

These data seem inconsistent with the findings of the oral studies in non-bile-cannulated rats where urinary excretion in males was 64 % of the AD and faecal elimination approximately 27 % of the AD. It is possible that in non-bile-cannulated animals, material excreted in bile is re-absorbed leading to post-hepatic re-circulation. However, a more appropriate comparison of the urinary and faecal excretion values between cannulated and non-cannulated animals would have been possible if an oral bile cannulated study had been performed. HSE is of the view that extrapolation of the biliary excretion data determined in this IV study to the oral route of administration does not seem appropriate as the kinetics (and in particular the biliary excretion) of bixlozone following IV administration are likely to be different from those following oral administration. On this basis, the biliary excretion data determined in this study will not be used to calculate a more accurate value of oral absorption.

Metabolite profiles in bile and urine (

Table B 6.1.2.2*)*

The radioactive peak distribution and their equivalent in percentage of AD were calculated from the pooled bile and urine samples; this was not conducted in faeces samples since only ≤ 1% of the AD was recovered in faeces collected for 5 days. In order to release the conjugate forms of metabolites and optimise the detection of conjugated metabolites of similar polarity eluting together, the bile and urine samples were also subjected to enzyme hydrolysis.

The metabolic profiles of the pooled bile samples at 0-8 and 8-24 hours showed that 5-OH-bixlozone -glucuronide as the predominant metabolite, representing > 75 % of the radioactivity in bile and accounted for about 42 % of the AD; this metabolite was also found in urine in this study and in the excretion and metabolism study conducted with [^{14}C-Phenyl]-bixlozone (Section B.6.1.1.5). The metabolites di-OH- isobutyramide-bixlozone glucuronide, and bixlozone -ring-open oxidative product (Mw259, B9) were detected in lesser amounts, each representing < 10 % of bile radioactivity and accounting for < 5 % of the AD. Bixlozone-dimethyl malonamide and 4-OH-Me-bixlozone were also found at < 5 % of the AD after enzyme hydrolysis. The unchanged parent compound bixlozone was only detected at low levels (< 1 % of the AD).

In urine, the metabolite 5-OH- bixlozone-glucuronide was also found at levels ≥ 10 % of the AD, whilst 5-keto-hydrate-bixlozone accounted for 8 % of the AD. The metabolites 2,4-dichlorohippuric acid, di-OH-isobutyramide- bixlozone-glucuronide, di-OH-isobutyramide-bixlozone, bixlozone-dimethyl malonamide, 4-OH-Me-bixlozone, and 5-OH-bixlozone were detected in relatively lesser amounts, each representing < 5 % the AD. As in bile, the unchanged parent compound was detected at low level in urine (< 1 % of the AD). No unique metabolite was identified in this IV study when compared to the oral metabolism study.

Overall 5-OH bixlozone (found predominantly as a conjugated glucuronide) was the most prominent metabolite found in bile and urine.

Table B 6.1.2.2: Percent of administered dose of bixlozone and its metabolites before and following enzyme hydrolysis of 0-24 hour pooled bile and urine samples from bile duct cannulated male rats administered a single 3 mg/kg bw IV dose of [^{14}C-phenyl]-bixlozone

Metabolite name	MS ID name	RT (min)	Metabolite #	Bile % of AD		Urine % of AD		Total % of AD before hydrolysis	Total % of AD after hydrolysis
				Before Hydrolysis	After Hydrolysis	Before Hydrolysis	After Hydrolysis		
Unknown	NA	13.0 - 13.2	B1	1.44	1.4	ND	ND	1.44	1.4
Unknown	NA	13.5 - 14.6	B2	0.88	0.8	ND	ND	0.88	0.8
Unknown	NA	15.3 - 15.5	B3	0.56	ND	ND	ND	0.56	ND
2,4-Dichlorohippuric acid	Mw247	16.6 - 18.0	U1	ND	ND	2.73	2.0	2.73	2.0
Di-OH-isobutyramide-bixlozone - glucuronide	Mw453	19.6 - 20.6	B4 / U2	4.51	1.0	4.07	0.4	8.6	1.3
5-OH-bixlozone-glucuronide	Mw465	23.3 - 24.2	B5 / U3	41.73	1.6	19.96	1.5	62	3.1
5-Keto-hydrate-bixlozone	Mw305	27.2 - 27.6	U4	ND	ND	10.44**	8.2	10.44	8.2
Unknown	NA	28.2 - 28.5	U5	ND	ND		1.4		1.4
Di-OH-isobutyramide-bixlozone	Mw277	28.9 - 29.2	B6 / U6	ND	4.3		4.4		8.6
bixlozone-dimethyl malonamide	Mw289a	31.1 - 31.2	B7 / U7	Trace	< 0.4	Trace	0.4	Trace	0.4
4-OH-Me-bixlozone	Mw289b	32.4 - 32.7	B8 / U8	ND	1.6	0.20	1.0	0.2	2.6
bixlozone-ring-open oxidative product	Mw259	34.1 - 34.6	B9	2.03	2.6	ND	ND	2.03	2.6
5-OH-bixlozone	Mw289c	34.6 - 34.8	B10 / U9	Trace	38.4	0.54	18.1	0.54	56.5
Bixlozone	Mw273	37.7 - 39.2	TS (B11, U10)	0.47	ND	0.42	0.9	0.9	0.9
Total identified (%)									**86.3**

NA: not available; ND: not detected

*B9 and B10 co-eluent. The percentage of B9 assumed to keep consistent before and after hydrolysis. The integration of B9 + B10 is 79.33 %, then B10 = 79.33 – 5.01 = 74.32 %.

** U5 and U6 were minor peaks and detected as the shoulder peaks of U4 in the 8-24 hr pooled sample. Therefore the % dose for 0-24 hours is presented for U4 + U5 + U6

Conclusion

In a non-GLP study in bile duct-cannulated male rats, administered a single IV dose of [^{14}C-Phenyl]-bixlozone at 3 mg/kg bw, bixlozone and/or its metabolites were rapidly excreted via the bile and urine (52 and 40 % of the

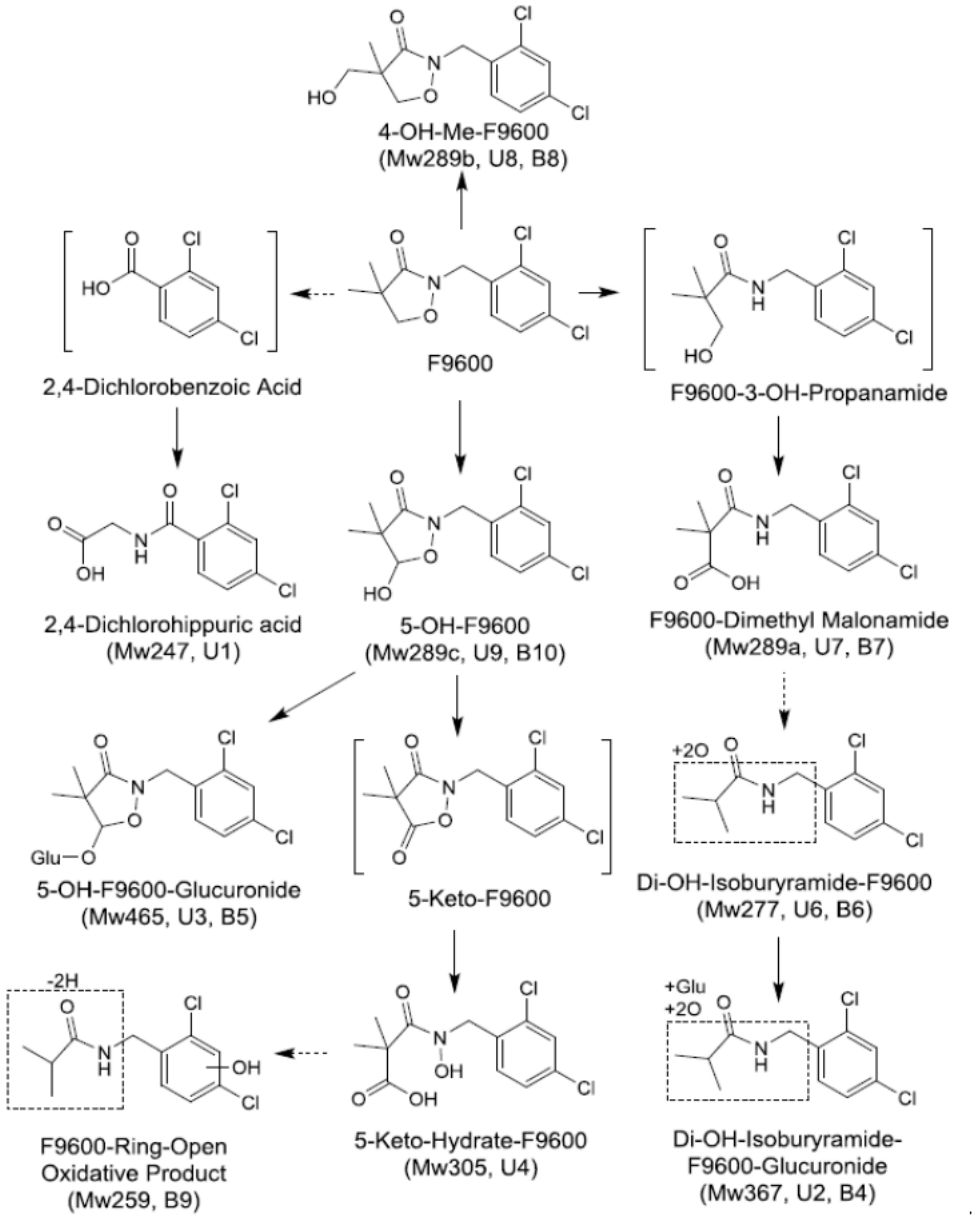
administered dose, respectively). In excess of 90 % of the AD was recovered within 5 days post-dose. The metabolic profiles showed that [^{14}C-Phenyl]-bixlozone was extensively metabolised in rats. In the bile, the predominant metabolite was 5-OH-bixlozone-glucuronide; whilst in urine, the metabolites 5-OH-bixlozone - glucuronide and 5-keto-hydrate-bixlozone were the most prevalent. Minimal radioactivity, (approximately 1 % of the AD), was excreted through faeces; therefore gastric secretion was not significant.

This study showed that [^{14}C-Phenyl]-bixlozone was extensively metabolised in bile duct cannulated male rats, with oxidation (hydroxylation), ring-opening, and glucuronidation of oxidative products, constituting the major metabolic pathways of bixlozone. No metabolite unique to the method of administration employed in this study (IV) was identified.

It is unclear to HSE why bile-cannulation was performed following IV administration rather than oral administration. As the kinetics (and in particular the biliary excretion) of bixlozone following IV administration are likely to be different from those following oral administration, HSE is of the view that the biliary and urinary excretion profiles determined from this study cannot be directly extrapolated to the oral route.

[redacted] (2017d)

Figure B 6.1.2.1: Proposed metabolic pathways of bixlozone in bile duct cannulated male rats following a single IV dose of [^{14}C-Phenyl]- bixlozone at 3 mg/kg bw



B.6.1.3. Comparative interspecies *in vitro* metabolism

Two comparative interspecies *in vitro* metabolism studies are available for bixlozone. The first one aimed to compare the *in vitro* metabolic profiles of [^{14}C-U-Phenyl]-bixlozone and [^{14}C-Carbonyl]-bixlozone in rat, mouse, dog and human hepatocytes (males and females) and identify potential unique human metabolite(s) or sex differences in the metabolism of bixlozone. From this study HSE observed that a disproportionate production of 4-OH-Me-bixlozone was seen in human hepatocytes compared to the other species, especially the rat and dog where it was not detected. Moreover, the study was concluded not to be GLP compliant following an audit, although HSE concluded that the shortcomings of the study did not compromise the validity of the study for regulatory purposes. In order to address these concerns, the applicant submitted a second study which is GLP compliant and used the same species (but was conducted with mixed-sex hepatocytes) and also a position statement to address the toxicological relevance of the disproportionate production of 4-OH-Me-bixlozone seen in human hepatocytes compared to the other species.

B.6.1.3.1. *Comparative in vitro metabolism of [^{14}C]-bixlozone (Phenyl and Carbonyl - labelled) in male and female mouse, rat, dog and human hepatocytes*

The aim of this study was to compare the *in vitro* metabolic profiles of [^{14}C-U-Phenyl]-bixlozone and [^{14}C-Carbonyl]-bixlozone in rat, mouse, dog and human hepatocytes and identify potential unique human metabolite(s) or sex differences in the metabolism of bixlozone. The species rat, mouse and dog were chosen because they were all used in the toxicological studies with bixlozone. The time point selected for sample collection (4 hours post-dose) was based on the T_{max} determined in a previous pharmacokinetic study (Please refer to study Report FMC-R3773 by [redacted] 2016).

Study # 10	Comparative *in vitro* Metabolism of [^{14}C] F9600 (Phenyl and Carbonyl-labelled) in mouse, rat, dog and human hepatocytes
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2017e)
Date performed	Jan 2016 – May 2017
Test facility	[redacted]
Report reference	Study no. FMC-R4547
Guideline(s)	N/A
Deviations from the guideline	N/A
GLP	No. The study is claimed to be GLP compliant however the US Environmental Protection Agency (US EPA) was requested to conduct a GLP compliance inspection and an audit on this study. It was concluded that "The Quality Assurance Unit did not submit the inspection report to the study director and test facility management in a timely manner. Further, there is insufficient coverage by the Quality Assurance as only the draft final report and raw data were audited and as inspections during study performance were lacking." Therefore, the study was concluded not to be GLP compliant. Nevertheless, HSE concludes that the shortcomings highlighted above are unlikely to compromise the scientific validity of the study and therefore this study remains acceptable for regulatory purposes.
Test material	[^{14}C-Phenyl]-F9600 Batch 77874-3-19; purity 100 %; specific activity 56 mCi/mmol (202.9 µCi/mg) [^{14}C-Carbonyl]-F9600 Batch CFQ42476; purity 99.9 %; specific activity 59 mCi/mmol (213.8 µCi/mg) F9600 Batch G3773-17; purity 99.5 %.
Study acceptable	Yes

Material and Methods

Pools of cryopreserved hepatocytes from male and female Sprague Dawley rat, CD-1 mouse, Beagle dog and human (cell viability > 85 %) were prepared as duplicate suspensions and were incubated at 37°C and 5 % CO_2 with 20 µM of [^{14}C-Phenyl]-bixlozone or [^{14}C-Carbonyl]-bixlozone for 0, 1, 2, and 4 hours. Following incubation, the collected samples were centrifuged, and the supernatants extracted using acetonitrile. The metabolite profiles were determined by HPLC with radioactivity detection. The chemical structures of major radioactive metabolites were identified/characterized by LC/MS methods and/or comparison with 16 synthetic

reference standards. To confirm the identification of glucuronide conjugates of primary metabolites detected in hepatocyte samples, 5 OH-bixlozone and 4-OH-Me-bixlozone standards were incubated separately with male rat liver microsomes in the presence of cofactor Uridine diphosphoglucuronic acid (UDPGA) at 37 ºC for 1 hour. Control incubations without (UDPGA) were also conducted at the same time.

The HPLC recovery of radioactivity was determined for one sample per species by counting the total amount of radioactivity in the eluent collected during a HPLC run (with column) compared to the total radioactivity in the eluent collected (without column) by Liquid scintillation counter (LSC).

Representative radio chromatograms of [^{14}C-Phenyl]-bixlozone and [^{14}C-Carbonyl]-bixlozone incubated in hepatocyte incubation medium without hepatocytes at 37 °C for 4 hours were prepared to show the stability of the test compounds. The hepatocytes' metabolic activity was confirmed by incubating them with 100 µM testosterone for approximately 4 hours under similar incubation conditions used for [^{14}C]-bixlozone. At the end of the incubation period the samples were processed using the same method described above.

Results (from Table B 6.1.3.1 to Table B 6.1.3.8)

[^{14}C-Phenyl]-bixlozone and [^{14}C-Carbonyl]-bixlozone were shown to be stable after 4 hours incubation in hepatocyte incubation medium without hepatocytes at 37°C. All batches of hepatocytes used in this study metabolised the positive control testosterone and thus were adequate for conducting the *in vitro* metabolism study.

The recovery of radioactivity from each incubation ranged between 87 % and 119 % of the applied radioactivity (AR), with a mean value (n = 16) of about 104 %. Greater than 90 % of the radioactivity injected onto HPLC was identified by LC/MS and/or by comparison with synthetic standards.

In general, bixlozone was extensively metabolised in hepatocytes; in rat and dog hepatocytes, both [^{14}C-Phenyl]-bixlozone and [^{14}C-Carbonyl]-bixlozone were virtually completely metabolised after 4 hour incubation, whilst the extent of metabolism of bixlozone in the mouse and human hepatocytes was about 56-69 % and 62-86 % of the AR, respectively.

A total of 13 metabolite peaks (excluding bixlozone) were found in the different hepatocyte incubations. The major metabolites identified for all species were 5-OH-bixlozone (unconjugated and / or as glucuronide conjugates) and 5-keto-hydrate- bixlozone (Table B 6.1.3.9).

In human hepatocytes, 4-OH-Me-bixlozone (unconjugated and / or as glucuronide conjugate) was another major metabolite identified, whilst 4-COOH-Me-bixlozone, the oxidative metabolite of 4-OH-Me-bixlozone, was a minor metabolite. The two metabolites were present in dog hepatocytes at levels < 10% of the AR but were not found in the rat; 4-OH-Me-bixlozone could be found in mouse samples at levels < 10 % of the AR. In rat hepatocytes, additional major metabolites were identified as carbamic acid and di-OH-isobutyramide bixlozone, which were detected as minor metabolites in mouse hepatocytes but not in dog or human samples.

The metabolite profiles retrieved from male hepatocytes were qualitatively similar to those retrieved from female hepatocytes in all species although the extent of metabolism seemed higher in the female samples compared to the male samples. Overall, there was no significant sex differences noted. Moreover, no label specific metabolites were observed in human hepatocytes.

Table B 6.1.3.1: Percent distribution of [^{14}C-Phenyl]-bixlozone and metabolites observed in male and female rat hepatocytes following incubations at 37°C for up to 4 hours

HPLC Peak No.	RT (min)	Metabolite Name	Percent distribution of [^{14}C-Phenyl]-bixlozone and metabolites observed rat hepatocytes (% of the AR)							
			0 Hour		1 Hour		2 Hour		4 Hour	
			Male	Female	Male	Female	Male	Female	Male	Female
M3	8.2-8.9	Unknown-2	ND	ND	3.85	3.14	5.02	5.68	4.82	4.29
M4	11.0-12.2	Glucuronide of 5-OH-bixlozone (Mw465a-1)	ND	ND	3.77	2.50	8.36	5.28	6.28	10.03
M5	12.5-13.7	Glucuronide of 5-OH-bixlozone (Mw465a-2)	ND	ND	33.10	27.94	30.27	44.79	45.68	56.72
M9	15.5-16.7	5-Keto Hydrate bixlozone (Mw305)	ND	ND	21.15	10.79	25.00	16.48	28.57	18.45
M10	16.7-17.7	Di-OH-Isobutyramide bixlozone (Mw277)	ND	ND	9.36	11.59	15.72	11.12	8.28	3.24
M11	18.0-19.3	4-OH-Me-bixlozone (Mw289a)	ND	ND	1.81	ND	2.17	ND	ND	ND
M12	20.3-21.2	5-OH bixlozone (Mw289b)	ND	7.31	4.87	9.34	3.51	4.73	6.37	7.28
TS	31.5-32.4	Bixlozone (Mw273)	100.00	92.69	22.09	34.70	9.95	11.91	ND	ND
Total identified peaks			100.00	100.00	96.15	96.86	94.98	94.31	95.18	95.72
Total peaks			100.00	100.00	100.00	100.00	100.00	99.99	100.00	100.01

AR: Applied Radioactivity
ND: Not detected.

Table B 6.1.3.2: Percent distribution of [^{14}C-Phenyl]-bixlozone and metabolites observed in male and female mouse hepatocytes following incubations at 37°C for up to 4 hours.

HPLC Peak No.	RT (min)	Metabolite Name	Percent distribution of [^{14}C-Phenyl]- bixlozone and metabolites observed mouse hepatocytes (% of the AR)							
			0 hour		1 hour		2 hours		4 hours	
			M	F	M	F	M	F	M	F
M4	11.0-12.2	Glucuronide of 5-OH-bixlozone (Mw465a-1)	ND	ND	2.95	6.04	2.79	6.88	2.78	4.00
M5	12.5-13.7	Glucuronide of 5-OH-bixlozone (Mw465a-2)	ND	ND	15.48	15.33	14.42	24.85	17.50	14.86
M8	14.2-14.5	Unknown-3	ND	ND	ND	ND	ND	2.45	ND	ND
M9	15.5-16.7	5-Keto Hydrate-bixlozone (Mw305)	ND	ND	13.03	10.22	11.53	16.10	17.84	19.06
M10[#]	16.7-17.7	Di-OH- Isobutyramide bixlozone (Mw277)	ND	ND	3.46	2.67	3.26	3.50	4.40	4.30
M11	18.0-19.3	4-OH-Me-bixlozone (Mw289a)	ND	ND	4.68	ND	6.70	1.28	3.36	4.00
M12	20.3-21.2	5-OH-bixlozone (Mw289b)	7.98	12.21	9.47	20.56	12.65	17.74	10.54	16.09
M13	21.1-22.6	4-COOH-bixlozone (Mw303)	ND	ND	ND	ND	ND	3.27	ND	ND
TS	31.5-32.4	Bixlozone (Mw273)	92.02	87.79	50.92	45.18	48.65	23.92	43.57	37.70
Total identified peaks			100.00	100.00	100.00	100.00	100.00	97.54	100.00	100.01
Total peaks			100.00	100.00	100.00	100.00	100.00	99.99	100.00	100.01

AR: Applied Radioactivity
ND: Not detected.
M: males; F: females; ND: Not detected.
[#]M10 identified based on HPLC retention time.

Table B 6.1.3.3: Percent distribution of [^{14}C-Phenyl]-bixlozone and metabolites observed in male and female dog hepatocytes following incubations at 37°C for up to 4 hours

HPLC Peak No.	RT (min)	Metabolite Name	Percent distribution of [^{14}C-Phenyl]- bixlozone and metabolites observed dog hepatocytes (% of the AR)							
			0 Hour		1 Hour		2 Hour		4 Hour	
			Male	Female	Male	Female	Male	Female	Male	Female
M4	11.0-12.2	Glucuronide of 5-OH-bixlozone (Mw465a-1)	ND	ND	11.64	44.49	66.20	66.17	64.56	64.78
M6 (M7)	12.9-13.5	Glucuronide of 4-OH-Me-bixlozone (Mw465b-1/Mw465b-2)	ND	ND	ND	1.85	1.96	2.73	3.48	2.78
M8	14.2-14.5	Unknown-3	ND	ND	1.89	4.05	1.68	ND	ND	ND
M9	15.5-16.7	5-Keto Hydrate bixlozone (Mw305)	ND	ND	6.39	13.3	24.86	24.98	26.21	25.74
M11	18.0-19.3	4-OH-Me-bixlozone (Mw289a)	ND	ND	6.23	4.23	ND	ND	ND	ND
M12	20.3-21.2	5-OH-bixlozone (Mw289b)	ND	ND	47.30	28.72	ND	ND	ND	ND
M13	21.1-22.6	4-COOH-bixlozone (Mw303)	ND	ND	ND	ND	5.31	6.12	5.74	6.70
TS	31.5-32.4	Bixlozone (Mw273)	100.00	100.00	26.56	3.35	ND	ND	ND	ND
Total identified peaks			100.00	100.00	98.12	95.95	100.00	100.00	99.99	100.00
Total peaks			100.00	100.00	100.00	100.00	100.00	100.00	99.99	100.00

AR: Applied Radioactivity
ND: Not detected.
*M6 and M7 co-eluted in the method used in this study.

Table B 6.1.3.4: Percent distribution of [^{14}C-Phenyl]- bixlozone and metabolites observed in male and female human hepatocytes following incubations at 37°C for up to 4 hours

HPLC Peak No.	RT (min)	Metabolite Name	Percent distribution of [^{14}C-Phenyl]- bixlozone and metabolites observed human hepatocytes (% of the AR)							
			0 Hour		1 Hour		2 Hour		4 Hour	
			Male	Female	Male	Female	Male	Female	Male	Female
M4	11.0-12.2	Glucuronide of 5-OH-bixlozone (Mw465a-1)	ND	ND	ND	2.50	4.16	6.02	8.53	9.39
M6 (M7)	12.9-13.5	Glucuronide of 4-OH-Me-bixlozone (Mw465b-1/Mw465b-2)	ND	ND	ND	ND	4.70	7.68	14.36	23.34
M9	15.5-16.7	5-Keto Hydrate bixlozone (Mw305)	ND	ND	5.10	7.92	9.21	17.36	16.01	26.00
M11	18.0-19.3	4-OH-Me-bixlozone (Mw289a)	ND	ND	7.95	14.99	17.43	21.03	20.54	20.93
M13	21.1-22.6	4-COOH-bixlozone (Mw303)	ND	ND	ND	ND	ND	ND	2.26	3.32
TS	31.5-32.4	Bixlozone (Mw273)	100.00	100.00	86.95	74.59	64.50	47.91	38.29	17.03
Total identified peaks			100.00	100.00	100.00	100.00	100.00	100.00	99.99	100.01
Total peaks			100.00	100.00	100.00	100.00	100.00	100.00	99.99	100.01

AR: Applied Radioactivity
ND: Not detected.
*M6 and M7 co-eluted in the method used in this study.

Table B 6.1.3.5: Percent distribution of [^{14}C-Carbonyl]-bixlozone and metabolites observed in male and female rat hepatocytes following incubations at 37°C for up to 4 hours

HPLC Peak No.	RT (min)	Metabolite Name	Percent distribution of [^{14}C-Carbonyl]-bixlozone and metabolites observed rat hepatocytes (% of the AR)							
			0 Hour		1 Hour		2 Hour		4 Hour	
			Male	Female	Male	Female	Male	Female	Male	Female
M1	2.9-3.4	Carbamic Acid	ND	ND	7.13	5.30	11.60	7.31	14.93	9.03
M3	8.2-8.9	Unknown-2	4.63	ND	6.97	2.89	7.87	6.04	3.58	4.44
M4	11.0-12.2	Glucuronide of 5-OH-bixlozone (Mw465a-1)	ND	ND	1.36	ND	1.33	ND	ND	1.36
M5	12.5-13.7	Glucuronide of 5-OH-bixlozone (Mw465a-2)	ND	ND	34.72	27.85	43.91	51.81	47.34	64.41
M9	15.5-16.7	5-Keto Hydrate bixlozone (Mw305)	ND	ND	19.94	8.97	20.71	14.97	22.09	13.52
M10	16.7-17.7	Di-OH-Isobutyramide bixlozone (Mw277)	ND	ND	14.56	8.42	3.89	8.29	ND	ND
M12	20.3-21.2	5-OH-bixlozone (Mw289b)	17.50	10.11	5.99	10.14	10.69	6.74	12.07	7.25
TS	31.5-32.4	Bixlozone (Mw273)	77.86	89.89	9.33	36.43	ND	4.84	ND	ND
Total identified peaks			95.36	100.00	93.03	97.11	92.13	93.96	96.43	95.57
Total peaks			99.99	100.00	100.00	100.00	100.00	100.00	100.00	100.00

AR: Applied Radioactivity
ND: Not detected.

Table B 6.1.3.6: Percent distribution of [^{14}C-Carbonyl]-bixlozone and metabolites observed in male and female mouse hepatocytes following incubations at 37°C for up to 4 hours

HPLC Peak No.	RT (min)	Metabolite Name	Percent distribution of [^{14}C-Carbonyl]-bixlozone and metabolites observed mouse hepatocytes (% of the AR)							
			0 Hour		1 Hour		2 Hour		4 Hour	
			Male	Female	Male	Female	Male	Female	Male	Female
M1	2.9-3.4	Carbamic Acid	ND	ND	ND	ND	ND	2.18	2.14	2.50
M2	5.2	Unknown-1	ND	ND	ND	ND	ND	3.79	ND	ND
M3	8.2-8.9	Unknown-2	ND	ND	ND	3.02	2.95	4.55	2.45	2.39
M4	11.0-12.2	Glucuronide of 5-OH-bixlozone (Mw465a-1)	ND	ND	3.17	6.23	5.22	6.06	5.71	4.02
M5	12.5-13.7	Glucuronide of 5-OH-bixlozone (Mw465a-2)	ND	ND	16.1	15.58	24.31	27.18	24.29	17.28
M9	15.5-16.7	5-Keto Hydrate-bixlozone (Mw305)	ND	ND	7.48	10.52	13.29	16.00	15.10	20.00
M10#	16.7-17.7	Di-OH-Isobutyramide-bixlozone (Mw277)	ND	ND	ND	ND	ND	ND	1.94	ND
M11	18.0-19.3	4-OH-Me-bixlozone (Mw289a)	ND	ND	2.95	ND	3.64	2.37	2.45	3.48
M12	20.3-21.2	5-OH-bixlozone (Mw289b)	11.66	14.84	15.19	20.45	13.78	22.35	12.04	20.00
TS	31.5-32.4	Bixlozone (Mw273)	88.34	85.16	55.10	44.21	36.81	15.53	33.88	30.33
Total identified peaks			100.00	100.00	99.99	96.99	97.05	91.67	98.06	97.61
Total peaks			100.00	100.00	99.99	100.01	100.00	100.01	100.00	100.00

AR: Applied Radioactivity
ND: Not detected.
#M10 identified based on HPLC retention time.

Table B 6.1.3.7: Percent distribution of [^{14}C-Carbonyl]-bixlozone and metabolites observed in male and female dog hepatocytes following incubations at 37°C for up to 4 hours

HPLC Peak No.	RT (min)	Metabolite Name	Percent distribution of [^{14}C-Carbonyl]-bixlozone and metabolites observed dog hepatocytes (% of the AR)							
			0 Hour		1 Hour		2 Hour		4 Hour	
			Male	Female	Male	Female	Male	Female	Male	Female
M4	11.0-12.2	Glucuronide of 5-OH-bixlozone (Mw465a-1)	ND	ND	11.01	48.48	63.11	68.20	61.72	63.02
M6 (M7)	12.9-13.5	Glucuronide of 4-OH-Me-bixlozone (Mw465b-1/Mw465b-2)	ND	ND	ND	3.66	4.03	4.67	4.40	4.75
M9	15.5-16.7	5-Keto Hydrate bixlozone (Mw305)	ND	ND	5.12	15.15	26.51	21.97	26.12	24.45
M11	18.0-19.3	4-OH-Me-bixlozone (Mw289a)	ND	ND	6.38	4.57	ND	ND	ND	ND
M12	20.3-21.2	5-OH bixlozone (Mw289b)	ND	ND	48.25	28.13	ND	ND	ND	ND
M13	21.1-22.6	4-COOH-bixlozone (Mw303)	ND	ND	ND	ND	6.35	5.17	7.76	7.78
TS	31.5-32.4	Bixlozone (Mw273)	100.00	100.00	29.24	ND	ND	ND	ND	ND
Total identified peaks			11.0-12.2	100.00	100.00	99.99	100.00	100.01	100.00	100.00
Total peaks			100.00	100.00	100.00	99.99	100.00	100.01	100.00	100.00

AR: Applied Radioactivity
ND: Not detected.
*M6 and M7 co-eluted in the method used in this study.

Table B 6.1.3.8: Percent distribution of [^{14}C-Carbonyl]-bixlozone and metabolites observed in male and female human hepatocytes following incubations at 37°C for up to 4 hours

HPLC Peak No.	RT (min)	Metabolite Name	Percent distribution of [^{14}C-Carbonyl]-bixlozone and metabolites observed human hepatocytes (% of the AR)							
			0 Hour		1 Hour		2 Hour		4 Hour	
			Male	Female	Male	Female	Male	Female	Male	Female
M4	11.0-12.2	Glucuronide of 5-OH-bixlozone (Mw465a-1)	ND	ND	1.41	2.55	4.34	4.10	9.92	9.78
M6 (M7)	12.9-13.5	Glucuronide of 4-OH-Me-bixlozone (Mw465b-1/Mw465b-2)	ND	ND	ND	ND	4.23	7.56	16.42	27.48
M9	15.5-16.7	5-Keto Hydrate bixlozone (Mw305)	ND	ND	6.34	7.10	9.67	16.25	17.19	25.25
M11	18.0-19.3	4-OH-Me-bixlozone (Mw289a)	ND	ND	8.71	14.39	18.89	25.58	20.27	20.48
M13	21.1-22.6	4-COOH-bixlozone (Mw303)	ND	ND	ND	ND	ND	ND	1.45	3.08
TS	31.5-32.4	Bixlozone (Mw273)	100.00	100.00	83.54	75.96	62.87	46.50	34.73	13.93
Total identified peaks			100.00	100.00	100.00	99.99	99.98	100.00	100.00	100.00
Total peaks			100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

AR: Applied Radioactivity
ND: Not detected.
*M6 and M7 co-eluted in the method used in this study.

Regarding male and female human hepatocytes, all metabolites identified were also detected in at least one of the animal species used for toxicology studies, i.e. mouse, rat or dog. However, HSE notes that the metabolite 4-OH-Me-bixlozone (M11) was produced at much higher levels in human hepatocytes compared to the other

species, with no detection in rats and dogs and levels 5-8-times lower in mice. In addition, the glucuronide conjugate of 4-OH-Me-bixlozone was noted in the dog and human only, with a % of total radioactivity 4 to 8 times lower in the dog than in human hepatocytes.

A summary table presenting the percent distribution of bixlozone and its metabolites in hepatocytes from male and female mice, rats, dogs, and humans after a 4 hours incubation with [^{14}C-Phenyl]-bixlozone or [^{14}C-Carbonyl]-bixlozone is shown below:

Table B 6.1.3.9: Percent distribution of bixlozone and its metabolites in HPLC chromatograms of hepatocytes from male and female mice, rats, dogs, and humans after a 4 hours incubation with [^{14}C-Phenyl]-bixlozone or [^{14}C-Carbonyl]-bixlozone

Metabolite Names	% of the AR															
	Mouse				Rat				Dog				Human			
	Phenyl		Carbonyl		Phenyl		Carbonyl		Phenyl		Carbonyl		Phenyl		Carbonyl	
	M	F	M	F	M	F	M	F	F	M	F	M	M	F	M	F
Carbamic acid	-	-	2.1	2.5	-	-	14.9	9.0	-	-	-	-	-	-	-	-
Unknown-2	-	-	2.5	2.4	4.8	4.3	3.6	4.4	-	-	-	-	-	-	-	-
Glucuronide 1 of 5-OH-F9600	2.8	4.0	5.7	4.0	6.3	10.0	-	1.4	64.6	64.8	61.7	63.0	8.5	9.4	9.9	9.8
Glucuronide 2 of 5-OH-F9600	17.5	14.9	24.3	17.3	45.7	56.7	47.3	64.4	-	-	-	-	-	-	-	-
Glucuronide 1 of 4-OH-Me-F9600	-	-	-	-	-	-	-	-	**3.5**	**2.8**	**4.4**	**4.8**	**14.4**	**23.3**	**16.4**	**27.5**
Glucuronide 2 of 4-OH-Me-F9600	-	-	-	-	-	-	-	-								
5-Keto hydrate F9600	17.8	19.1	15.1	20.0	28.6	18.5	22.1	13.5	26.2	25.7	26.1	24.5	16.0	26.0	17.2	25.3
Di-OH-Isobutyramide F9600	4.4	4.3	1.9	-	8.3	3.2	-	-	-	-	-	-	-	-	-	-
4-OH-Me-F9600	**3.4**	**4.0**	**2.5**	**3.5**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**20.5**	**20.9**	**20.3**	**20.5**
5-OH-F9600	10.5	16.1	12.0	20.0	6.4	7.3	12.1	7.3	-	-	-	-	-	-	-	-
4-COOH-F9600	-	-	-	-	-	-	-	-	**5.7**	**6.7**	**7.8**	**7.8**	**2.3**	**3.3**	**1.5**	**3.1**
F9600	43.6	37.7	33.9	30.3	-	-	-	-	-	-	-	-	38.3	17.0	34.7	13.9

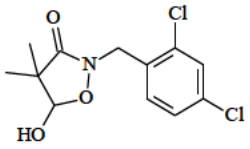
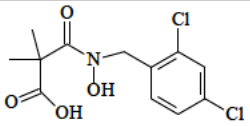
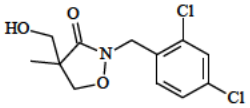
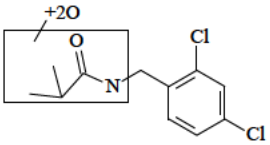
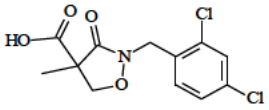
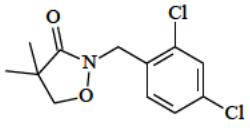
AR: Applied Radioactivity

Conclusion

In conclusion, the *in vitro* metabolic profiles of bixlozone were explored in rat, mouse, dog and human hepatocytes in this non-GLP study which is considered acceptable for regulatory purposes. Bixlozone was extensively metabolised in hepatocytes. [^{14}C]-bixlozone (20 µM) was virtually completely metabolised after incubation for 4 hours in rat and dog hepatocytes, and the extent of [^{14}C]-bixlozone conversion to metabolites was about 56-69 % of the AR in mouse hepatocytes and 62-86 % of the AR in human hepatocytes. The common metabolic reactions in all species tested included oxidation (hydroxylation) and conjugation (glucuronidation); the metabolic pathways drawn from the metabolism of bixlozone in hepatocytes are similar (but somewhat simplified) to those identified in rats after oral administration of [^{14}C-Phenyl]-bixlozone and [^{14}C-Carbonyl]-bixlozone (See Figure B 6.1.1.1 & Figure B 6.1.1.2). No unique metabolite was identified in human hepatocytes; however a disproportionate production of 4-OH-Me-bixlozone was observed in human hepatocytes compared to the other species, especially the rat and dog where it was not detected. In the mouse, levels 5-8-times lower were measured. In addition, the glucuronide form of the metabolite was found at higher levels in humans compared to dogs. The applicant has been asked to address the reliability and significance of this finding. No significant sex differences or label specific metabolites were observed in human samples.

A summary of the metabolites identified in the different species is presented below:

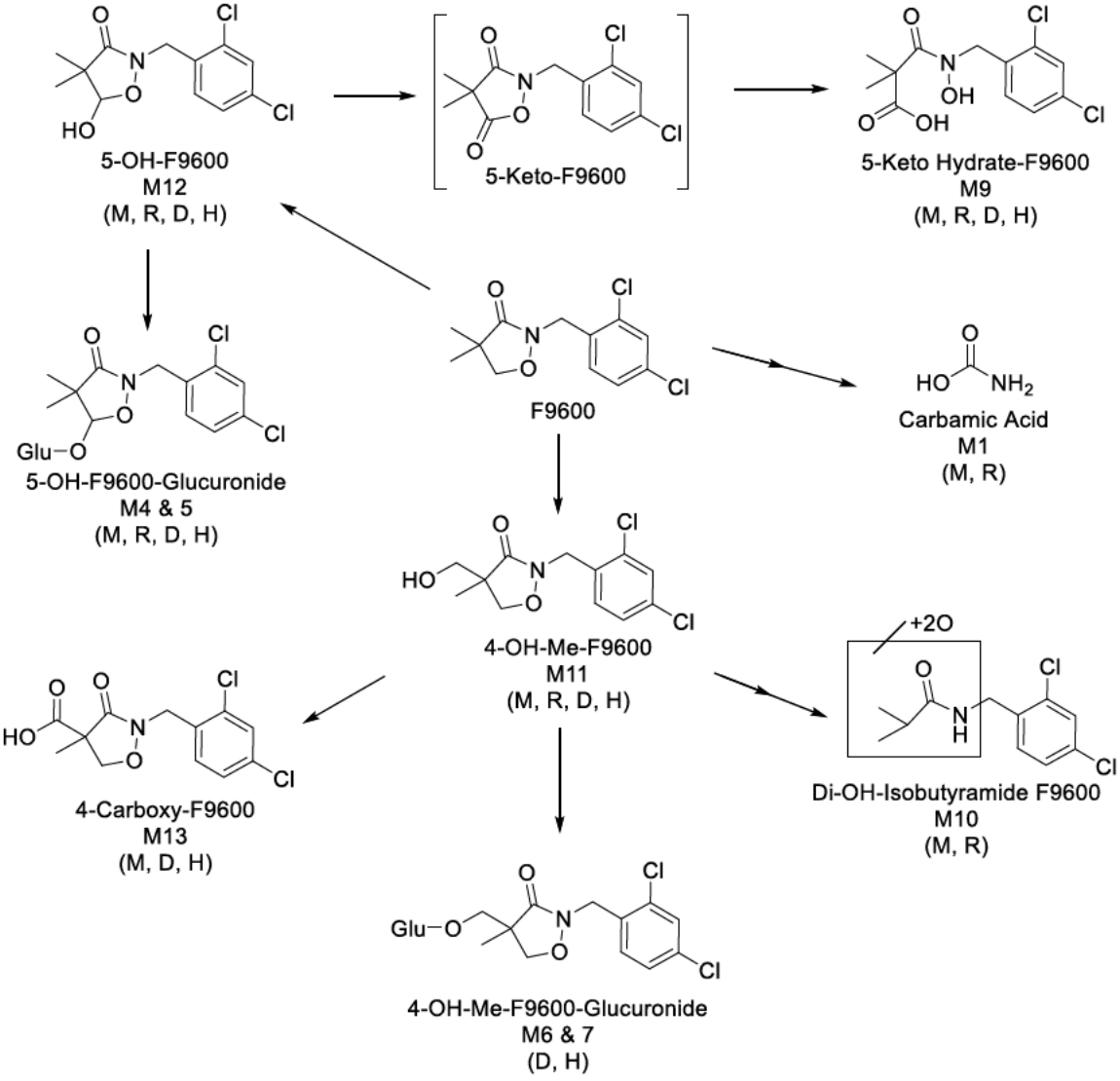
Table B 6.1.3.10: Metabolites identified in male and female rat, mouse, dog and human hepatocytes following incubation with [^{14}C-Phenyl]-bixlozone or [^{14}C-Carbonyl]-bixlozone for 4 hours at 37°C

Metabolite name	Structure	Biotransformation	Species Major / Minor
5-OH-bixlozone **		Oxidation (conjugation)	Rat, Mouse, Dog, Human Major metabolite for all species
5-Keto-hydrate-bixlozone		Oxidative ring-open metabolite	Rat, Mouse, Dog, Human Major metabolite for all species
4-OH-Me-bixlozone **		Oxidation (conjugation)	Human, major metabolite (approx. 20% with either label) Mouse (minor) (2.5-4 %) Dog (2.8-4.8 % glucuronide only) Rat (ND)
Di-OH-Isobutyramide bixlozone ***			Rat (minor) Mouse (minor) Dog (ND) Human (ND)
4-COOH-bixlozone		Oxidation	Mouse (minor) Dog (minor) Human (minor)
Bixlozone		Test compound	Mouse, Human Present after 4 hours incubation

ND = not detected
* Identification was based on HPLC retention time with synthetic standard
** Present as unconjugated form and/or glucuronide conjugates
* * * Characterized by retention time in mouse hepatocytes and by LC-MS in rat hepatocytes

The proposed *in vitro* metabolic pathways of bixlozone in rat, mouse, dog and human hepatocytes is presented below:

Figure B 6.1.3.1: Proposed metabolic pathways of bixlozone in rat, mouse, dog and human hepatocytes



[redacted] (2017e)

B.6.1.3.2. *Comparative in vitro metabolism of [^{14}C]-bixlozone (Phenyl and Carbonyl - labelled) in mixed-sex mouse, rat, dog and human hepatocytes*

The applicant submitted a second study conducted in the same laboratory as the first study: the study is GLP compliant, used the same methods as previously and hepatocytes from the same species selected for the first study; however mixed-sex hepatocytes were used instead of separated male and female hepatocytes.

Study # 11	In Vitro Comparative Metabolism of [14C-Phenyl]-and [14C-Carbonyl]-F9600 in Mixed Gender Mouse, Rat, Dog and Human Cryopreserved Hepatocytes
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2020)
Date performed	December 2019
Test facility	[redacted]
Report reference	Report no. FMC-53482

Guideline(s)	OECD 417 (2010)
Deviations from the guideline	None
GLP	Yes
Test material	[^{14}C-Phenyl]-F9600 Batch CFQ43508; purity 99.3 %; specific activity 61 mCi/mmol [^{14}C-Carbonyl]-F9600 Batch CFQ43509; purity 99.6 %; specific activity 56 mCi/mmol F9600 Batch PL14-0163; purity 99.8 %.
Study acceptable	Yes

Material and Methods

The Material and Methods employed for this study are identical to the previous study with the exception that pooled cryopreserved hepatocytes from mixed-sex mice, rats, dogs and humans were used this time. Please refer to the study by [redacted] (2017e) for more details.

Results (Table B 6.1.3.11**)**

[^{14}C-Phenyl]-bixlozone and [^{14}C-Carbonyl]-bixlozone were shown to be stable after 4 hours incubation in hepatocyte incubation medium without hepatocytes at 37°C. All batches of hepatocytes used in this study metabolised the positive control testosterone and thus were adequate for conducting the *in vitro* metabolism study.

The recovery of radioactivity from each incubation ranged between 84 % and 114 % of the AR, with a mean value (n = 18) of about 102 %. Greater than 90 % of the radioactivity injected onto HPLC was identified by LC/MS and/or by comparison with synthetic standards.

In general, bixlozone was extensively metabolised in hepatocytes; in dog hepatocytes, both [^{14}C-Phenyl]-bixlozone and [^{14}C-Carbonyl]-bixlozone were virtually completely metabolised after 4 hour incubation, whilst the extent of metabolism of bixlozone in the rat was 72-87 % of the AR, in the mouse about 86-92 % of the AR, and 49-51 % of the AR in human hepatocytes.

A total of 12 metabolite peaks (excluding bixlozone) were found in the different hepatocyte incubations. The metabolite profiles obtained after incubations with [^{14}C-Phenyl]-bixlozone or [^{14}C-Carbonyl]-bixlozone were qualitatively similar, except for the low molecular weight polar metabolite carbamic acid only found in the mouse and rat hepatocytes incubated with [^{14}C-Carbonyl]-bixlozone; the finding is consistent with the metabolic profile seen *in vivo* in the rat (please refer to Section B.6.1.1.6)

The major metabolite identified for all species was 5-keto-hydrate- bixlozone; 5-OH-bixlozone (unconjugated and / or as glucuronide conjugates) was also a major metabolite in the rat, mouse and dog hepatocytes but only a minor metabolite in human hepatocytes (Table B 6.1.3.11). This result is fairly consistent with the findings of the previous study.

In rat hepatocytes, carbamic acid and di-OH-isobutyramide bixlozone were only detected at low levels in this study compared to the previous study where they were identified as major metabolites; the difference could be explained by the reduced extent of metabolism in this study (72-87 % of the AR) compared to the previous study (≈100 % of the AR). These 2 metabolites were not found in dog or human samples.

In human hepatocytes, 4-OH-Me-bixlozone (unconjugated and / or as glucuronide conjugate) was another major metabolite identified, whilst 4-COOH-Me-bixlozone, the oxidative metabolite of 4-OH-Me-bixlozone, was a minor metabolite. The two metabolites were present in dog hepatocytes at levels < 10% of the AR but were not found in the rat except with [^{14}C-Phenyl]-bixlozone, where a very low level of 4-OH-Me-bixlozone was found; in mouse samples 4-OH-Me-bixlozone could be found at levels < 10 % of the AR.

Although no unique metabolite(s) or label specific metabolite(s) was (were) identified in human hepatocytes, a disproportionate production of 4-OH-Me-bixlozone was observed in human hepatocytes compared to the other species, especially for the rat where only a very low level of the glucuronide form of the metabolite was detected with the [^{14}C-Phenyl]-bixlozone. In the mouse and dog, several times lower levels of the metabolite were measured (unconjugated and / or as glucuronide conjugates) compared to the human samples.

A summary of the findings is presented in the table below:

Table B 6.1.3.11: Percent distributions of bixlozone and its metabolites in HPLC chromatograms of mixed-sex hepatocytes from rats, mice, dogs and humans incubated for 4 hours with [^{14}C-Phenyl]-bixlozone or [^{14}C-Carbonyl]-bixlozone

Metabolite names	% of the AR							
	Mouse		Rat		Dog		Human	
	Phenyl	Carbonyl	Phenyl	Carbonyl	Phenyl	Carbonyl	Phenyl	Carbonyl
Carbamic acid	-	0.68	-	1.02	-	-	-	-
Unknown	-	3.50	-	2.80	-	-	-	-
Glucuronide 1 of 5-OH-F9600	5.50	3.87	1.20	1.04	77.21	81.86	5.87	5.34
Glucuronide 2 of 5-OH-F9600	23.87	36.38	20.03	23.97	-	-	-	-
Glucuronide 1 of 4-OH-Me-F9600	**trace**	**1.52**	**0.57**	**trace**	**3.79**	**4.14**	**5.66**	**7.12**
Glucuronide 2 of 4-OH-Me-F9600	**trace**	**1.99**	**0.55**	**trace**	**3.31**	**2.48**	**3.25**	**5.52**
5-Keto-F9600 hydrate	30.47	24.91	22.02	31.19	13.02	7.58	17.12	14.53
Di-OH-Isobutyramide F9600	-	-	6.66	6.86	-	-	-	-
F9600-Dimethylmalonamide	2.09	1.31	2.96	2.91	-	-	-	-
4-OH-Me-F9600	**0.66**	**trace**	**Trace**	**trace**	-	-	**16.88**	**11.83**
5-OH-F9600	22.06	15.20	15.23	17.18	-	-	-	-
4-COOH-F9600	**trace**	**trace**	**Trace**	**trace**	**1.72**	**4.24**	**1.59**	**1.90**
F9600	13.75	7.62	27.6	13.4		-	48.96	50.56

Conclusion

In conclusion, the *in vitro* metabolic profiles of bixlozone were explored in rat, mouse, dog and human hepatocytes (mixed-sex) in a second GLP compliant study. Bixlozone was in general extensively metabolised in hepatocytes. [^{14}C]-bixlozone (20 µM) was virtually completely metabolised after incubation for 4 hours in dog hepatocytes, whilst the extent of metabolism of bixlozone in the rat was 72-87 % of the AR, in the mouse about 86-92 % of the AR, and 49-51 % of the AR in human hepatocytes. The common metabolic reactions in all species tested included oxidation (hydroxylation) and conjugation (glucuronidation); the metabolic pathways drawn from the metabolism of bixlozone in hepatocytes are similar (but somewhat simplified) to those identified in rats after oral administration of [^{14}C-Phenyl]-bixlozone and [^{14}C-Carbonyl]-bixlozone (See Figure B 6.1.1.1 & Figure B 6.1.1.2).

Overall the findings from this study were broadly similar to those of the previous *in vitro* comparative metabolism study. Small differences were seen in the rat samples but these were likely due to a lower extent in metabolism in this study (72-87 % of the AR) compared to the previous study (≈100 % of the AR). No unique or label-specific metabolite was identified in human hepatocytes; however, a disproportionate production of 4-OH-Me-bixlozone was again observed in human hepatocytes compared to the other species in this study. Thus, the applicant provided HSE with a position statement to address the toxicological relevance of the disproportionate production of 4-OH-Me-bixlozone seen in human hepatocytes compared to the other species, which is discussed further below.

The proposed *in vitro* metabolic pathways of bixlozone in rat, mouse, dog and human hepatocytes is similar to the pathways presented in the previous study (Figure B 6.1.3.1).

[redacted] (2020)

B.6.1.3.3. *Assessment of the toxicological relevance of the disproportionate production of 4-OH-Me-bixlozone seen in human hepatocytes compared to other species (rat, mouse & dog)*

The 2 *in vitro* comparative metabolism studies available for bixlozone showed a disproportionate production of 4-OH-Me-bixlozone in human hepatocytes compared to the other species, especially the rat and dog where it was not detected.

	Toxicological non-relevance of 4-hydroxymethyl-F9600 (4-OH-Me-F9600), a disproportionate in vitro human metabolite of Bixlozone
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	Wijeyesakere S.J., Wang W., Nallani G., Guo J. and Jackson S.A. (2020)
Date performed	March 2020
Test facility	FMC Corporation
Report reference	Report no. FMC-54077
Guideline(s)	N/A
Deviations from the guideline	None
GLP	N/A
Test material	N/A
Study acceptable	Yes, as part of the Weight of Evidence

The *in vitro* comparative animal metabolism studies with bixlozone followed all the recommendations of the EFSA workshop on *in vitro* comparative metabolism studies in regulatory pesticide risk assessment (EFSA Supporting publication 2019:EN-161) and met the study objective to determine comparative metabolic profiles of bixlozone across the species. Quantitative differences in the production of the metabolite 4-OH-Me-bixlozone exist between species, being present in human samples at disproportionately higher levels compared to other species (rat, mouse & dog).

The applicant evaluated the toxicological relevance of the disproportionate production of this metabolite. The evaluation considered the available toxicology data obtained on the parent compound bixlozone and includes a comparative genotoxicity (Q)SAR analysis of the metabolite compared to bixlozone, a comparison of physico-chemical properties and a structural similarity analysis.

Although there are no toxicity studies conducted with 4-OH-Me-bixlozone available, the applicant proposes that the toxicological data on bixlozone can be read-across based on the structural similarity of the two compounds. It should be noted that 4-OH-Me-bixlozone is bixlozone with an additional OH group on one of the carbonyl side-chain methyl groups (see Table 6.1.3.3.3).

Toxicology information on bixlozone

Bixlozone has a complete and robust toxicological data package showing it has low acute toxicity via the oral and dermal routes. Regarding acute toxicity via inhalation, HSE concluded that the available study was not fit for regulatory purposes and proposed, in the interest of animal welfare, to read-across from the study results of the structurally similar compound clomazone, concluding that bixlozone is acutely harmful by the inhalation route. Bixlozone is not a skin or eye irritant or a skin sensitiser. On repeated exposure, the liver was identified as the primary target organ of toxicity in the rat, mouse and dog. Bixlozone is carcinogenic in the mouse but it is not a reproductive toxicant. Bixlozone is not genotoxic based on the outcome of a battery of *in vitro* and *in vivo* studies. Furthermore, there is no evidence of neurotoxicity or endocrine disruption.

***In silico* assessment for genotoxicity**

The genotoxicity profile of the metabolite 4-OH-Me-bixlozone was evaluated computationally against several *in silico* tools and compared to the parent compound bixlozone, with the use of expert judgement and WoE approaches.

Assessment of the structures of bixlozone and 4-OH-Me-bixlozone revealed the lack of any structural features associated with chemical reactivity (defined as the ability of an electrophilic xenobiotic to covalently interact

with biological nucleophiles under physiological conditions without the need for enzymatic catalysis), a key molecular initiating event in the adverse outcome pathway for mutagenicity and clastogenicity (reviewed in [1,2])

Description of the (Q)SAR strategy

To predict the genotoxic potential (gene mutation and chromosomal aberrations) of 4-OH-Me-bixlozone, several models were used: ToxTree and Derek Nexus (Lhasa Ltd.) version 6.0.1 (Nexus: 2.2.2), which represent knowledge-based systems; and Sarah Nexus (Lhasa Ltd.) Model 2.0, a statistical tool. Further details on the model used can be found in Section **Error! Reference source not found.**.

(Q)SAR predictions results

The output from the above tools is summarised in the table below:

Table B 6.1.3.12: Summary of (Q)SAR predictions for bixlozone and 4-OH-Me-bixlozone

Chemical name	ToxTree	Derek Nexus			Sarah Nexus
	***In vitro* mutagenicity**	**Mutagenicity *in vitro***	**Chromosomal damage *in vitro***	**Chromosomal damage *in vivo***	**Mutagenicity**
Bixlozone (F9600; Parent)	No alerts	Inactive	No alert	No alert	Negative
4-OH-Me-bixlozone	No alerts	Inactive	No alert	No alert	Negative

There were no alerts for genotoxicity (mutagenicity or chromosomal damage) triggered by either the parent molecule (bixlozone) or 4-OH-Me-bixlozone. No unrecognised/uncharacterised structural features were identified by Derek Nexus for either bixlozone or 4-OH-Me-bixlozone.

Overall the predicted genotoxicity profile of 4-OH-Me-bixlozone is comparable to that of the parent compound bixlozone. In addition, since regulatory studies are available for bixlozone and showed this compound is not genotoxic, 4-OH-Me-bixlozone is also likely to be non-genotoxic.

Genotoxicity profiling using the OECD Toolbox version 4.3.1

Complementary (Q)SAR analysis was performed using the OECD Toolbox version 4.3.1. The molecular initiating events of relevance for this assessment involve interaction with DNA and/or proteins. The general mechanistic profilers included in the OECD Toolbox codifying the structural alerts of interest for assessment of these interactions are presented below and have been used for the assessment:

1. DNA binding by OASIS

2. DNA binding by OECD

3. Protein binding by OASIS

4. Protein binding by OECD

Additionally, the following endpoint specific profilers were run

1. DNA alerts for AMES by OASIS

2. DNA alerts for CA and MNT by OASIS

3. Protein binding alerts for chromosomal aberration by OASIS

4. *In vitro* mutagenicity (Ames test) alerts by ISS

5. *In vivo* mutagenicity (Micronucleus) alerts by ISS

No structural alerts were identified for the parent compound or 4-OH-Me-bixlozone using the following profilers: DNA binding by OASIS, DNA binding by OECD, Protein binding by OASIS, Protein binding by OECD, DNA alerts for AMES by OASIS, DNA alerts for CA and MNT by OASIS, Protein binding alerts for chromosomal aberration by OASIS and In vitro mutagenicity (Ames test) alerts by ISS.

[1] Benigni, R., and Bossa, C. (2011). Mechanisms of chemical carcinogenicity and mutagenicity: a review with implications for predictive toxicology. Chem Rev. 111(4):2507-2536. doi: 10.1021/cr100222q.

[2] Enoch, S.J., and Cronin, M,T. (2010). A review of the electrophilic reaction chemistry involved in covalent DNA binding. Crit Rev Toxicol. 40(8):728-748. doi:10.3109/10408444.2010.494175

There were alerts triggered for bixlozone and 4-OH-Me-bixlozone using the "*In vivo* mutagenicity (Micronucleus) alerts by ISS" profiler and they are presented in the table below:

Table B 6.1.3.13: Genotoxicity profiling of bixlozone and 4-OH-Me-bixlozone via the freely available OECD QSAR Toolbox.

Chemical name	Profiling in OECD Toolbox (v. 4.3.1) 5	Assessment of organic functional groups					
	in vivo mutagenicity (Micronucleus) alerts by ISS	Aryl	Aryl Halide	Benzyl	Oxazolidine derivatives	Saturated heterocyclic fragment	Alcohol
Bixlozone (F9600; parent)	H-acceptor-path3-H-acceptor	X	X	X	X	X	
4-OH-Me-bixlozone	H-acceptor-path3-H-acceptor	X	X	X	X	X	X

The presence of a putative intercalator or grove-binding moiety (H-acceptor-path3-H-acceptor) was identified by the ISS *in vivo* mutagenicity alerts profiler as an alert for *in vivo* mutagenicity (micronucleus formation) for both bixlozone and 4-OH-Me-bixlozone. This alert is unlikely to be biologically relevant since the predictive power of this alert has been suggested to be low by Benigni et al[3]. Moreover, an *in vivo* micronucleus assay is available for bixlozone and was clearly negative.

Overall, a single alert was triggered using the profilers in the OECD Toolbox which is unlikely to be biologically relevant.

Conclusion of the *in silico* genotoxicity assessment

Taking into consideration all the findings from the (Q)SAR tools, the results showed that 4-OH-Me-bixlozone did not trigger any additional alert for genotoxicity compared to the parent compound bixlozone. Since bixlozone is shown not to be genotoxic (Please refer to the summary on genotoxicity in Section B.6.4.4), 4-OH-Me-bixlozone is also unlikely to be genotoxic.

Read-across from Bixlozone (Table B 6.1.3.14**)**

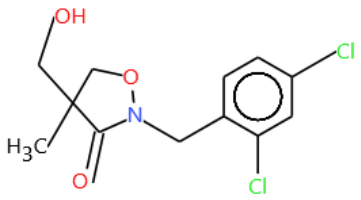
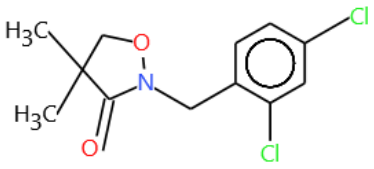
The metabolite of interest 4-OH-Me-bixlozone and bixlozone differ only by the presence of an additional single hydroxy group on a methyl group. All of the organic functional groups present in bixlozone are conserved in metabolite 4-OH-Me-bixlozone, which contains an extra alcohol function group absent in bixlozone (shown in table B.6.1.3.3.3).

4-OH-Me-bixlozone is structurally similar to the parent molecule bixlozone, with a dice similarity equal to 86 % calculated using the atom-centered hologram method within the OECD QSAR Toolbox. Furthermore, the 2 compounds share similar physical-chemical properties as shown in the table below. 4-OH-Me-bixlozine is more polar than the parent bixlozone. It is rapidly eliminated through oxidation to 4-COOH-Me-bixlozone and glucuronidation as demonstrated by the available *in vivo* rat studies (Section B.6.1.1.5).

Thus, it is reasonable to propose that the toxicity profile of 4-OH-Me-bixlozone comparable to that of the parent bixlozone.

[3] Benigni, R., Bossa, C., and Worth, A. (2012). Structural analysis and predictive value of the rodent *in vivo* micronucleus assay results. *Mutagenesis*. 25(4):335-341.

Table B 6.1.3.14: Structures and physical-chemical properties of 4-OH-Me-bixlozone and bixlozone (F9600)

Chemical Name	**4-hydroxymethyl F9600; 4-OH-Me-bixlozone; FMC-510234**	**Bixlozone (F9600) (CAS # 81777-95-9)**
Structure	 CC1(CO)CON(Cc2ccc(Cl)cc2Cl)C1=O	 CC1(C)CON(Cc2ccc(Cl)cc2Cl)C1=O
Structural (Dice) similarity to Bixlozone [a]	0.86	1.0
Log Kow	2.04	3.51
Acidic pKa [b]	10.5	10.7
Molecular weight (Da)	290	274

[a] Calculated via the atom-centered hologram method within the OECD QSAR Toolbox (version 4.3.1)
[b] Calculated via the OASIS Electric method within the OECD QSAR Toolbox (version 4.3.1)

Conclusion

4-OH-Me-bixlozone is a hydroxy metabolite of bixlozone which was found at disproportionate levels *in vitro* in human hepatocytes compared to the other species considered (mouse, rat and dog) and especially compared to rat hepatocytes. *In silico* genotoxicity comparative analysis, structural similarity analysis and a comparison of the physical-chemical properties of 4-OH-Me-bixlozone with those of the parent compound indicate the metabolite has a comparable toxicity profile to that of bixlozone. *In vivo* rat studies also showed the metabolite is rapidly eliminated in urine through oxidation to 4-COOH-Me-bixlozone and glucuronidation, suggesting that the metabolite is most likely less toxic than the parent substance. Therefore, the disproportionate production of this metabolite in human hepatocytes compared to the rat, our primary test species, is unlikely to lead to additional toxic effects beyond those already identified in the tested species as its toxicity profile is comparable to (and possibly less toxic than) that of the parent substance which has been fully tested in our model experimental animals.

B.6.1.4. Summary of ADME

The absorption, distribution, metabolism and excretion of bixlozone in mammals have been extensively investigated in Sprague-Dawley (SD) rats following a single oral low dose (5 mg/kg bw), a single oral high dose (500 mg/kg bw and 1000 mg/kg bw), multiple oral low doses (5 mg/kg bw, 14 days) and a single low IV dose (3 mg/kg bw) of [^{14}C-Phenyl]-bixlozone. Moreover, a mass balance and excretion study was conducted with [^{14}C-Carbonyl]-bixlozone at a single low dose (5 mg/kg bw). In addition to the *in vivo* studies, two *in vitro* metabolism studies of bixlozone using cryopreserved hepatocytes of rats, mouse, dog and human were performed. Lastly, additional limited toxicokinetic data from repeated dose and carcinogenicity studies conducted in rats, mice and dogs are available. The table below presents an overview of all the available studies.

Table B 6.1.4.1: Summary of the ADME studies of bixlozone

Method, Species, test substance, ***acceptability***	**Doses**	**Main findings**
Pharmacokinetics and metabolism (pilot study) **Rat** (Crl :CD9(SD), males & females, 3/sex/group) Not to GLP Not to OECD Guideline Deviations: N/A F9600 technical, batch G3773-17 Purity: 99.25 % Report no. FMC-R2838 [redacted] **(2014)** ***Supplementary only***	25 mg/kg bw Single oral (gavage)	One male was excluded from data analysis due to blockage of the catheter. Males (n=2): $T_{1/2}$ 3.11 h, T_{max} 0.5 h, C_{max} 169 ng/mL, AUC_{inf} 590 h ng/mL Females (n=3): $T_{1/2}$ 1.94 h, T_{max} 0.67 h, C_{max} 315 ng/mL, AUC_{inf} 982 h ng/mL Systemic exposure : 1.7 -1.9-fold greater for females compared to males. No F9600 detected in urine and rat faeces Extensive metabolism to various isoxazolidinone ring-opened/modified analogues in both urine and faeces.

Metabolism (pilot study) **Rat** (Crl :CD9(SD) 2 males Not to GLP OECD 417 (2010) Deviations: N/A **[^{14}C-phenyl]-F9600**, batch CFQ42017; purity 99.6 %; specific activity 56 mCi/mmol (202.9 µCi/mg) F9600 technical, batchPL14-0163; purity 99.8 % Study no. FMC-R3694 [redacted] **(2017a)** ***Supplementary only***	1000 mg/kg bw Single oral (gavage)	95 % of the dose was excreted in urine and faeces within 5 days with 72 % in urine and 24 % in faeces. Excretion through expired air was negligible. Bixlozone was extensively metabolised. Oxidation and ring-opening, followed by conjugation constituted the major metabolic reactions observed.

Toxicokinetics **Rat** (Crl :CD9(SD) 4/sex/group in all groups except for single oral high dose with 8/sex/group GLP OECD 417 (2010) Deviations: None of significance **[^{14}C-Phenyl]-F9600**, Batch CFQ42017; purity 99.6 %; specific activity 56 mCi/mmol (202.9 µCi/mg) F9600 technical, PL14-0163; purity 99.8 % Study no. FMC-P3773 [redacted]**(2016)** ***Acceptable***	Single oral low dose (5 mg/kg bw) Single oral high dose (1000 mg/kg bw) Multiple oral dose (5 mg/kg bw/day; 14 days) Intravenous bolus dose (IV; 3 mg/kg bw)	<u>Single oral low dose (5 mg/kg bw):</u> C_{max} of F9600 in plasma: 174 and 293 ng/mL at (Tmax) 0.25 h in male and female rats, respectively (M / F) $T_{1/2}$:1.4 h and 1.7 h in M / F $AUC_{0\text{-}inf}$: 145 and 221 ng x h/mL in M /F Bioavailability: 70 % and 86 % (total radioactivity) & 11 % and 18 % (F9600 in plasma) for M /F <u>Single oral high dose (1000 mg/kg bw):</u> C_{max} of F9600 in plasma: 9565 and 15060 ng/mL at (Tmax) 3.5 h in M / F $T_{1/2}$: 11 h and 14 h in M / F $AUC_{0\text{-}inf}$: $10.5x10^5$ and $35.9x10^5$ ng h/mL in M /F Bioavailability: 58 % and 60 % (total radioactivity) & 39 % and 100 % (F9600 in plasma) for M /F <u>Multiple oral dose (5 mg/kg bw/day; 14 days)</u> C_{max} of F9600 in plasma: 71 and 166 ng/mL in M / F at (Tmax) 0.0 h and 0.25 in M / F $T_{1/2}$: 11 h and 14 h in M / F $AUC_{0\text{-}inf}$: 65 and 162 ng h/mL in M /F Bioavailability: 58 % and 79 % (total radioactivity) & 5 % and 13 % (F9600 in plasma) for M /F No indications of accumulation of total radioactivity or F9600. <u>Intravenous bolus dose (IV; 3 mg/kg bw):</u> C_{max} of F9600 in plasma: 1317 and 1195 ng/mL at (Tmax) 0.08 h in M / F $T_{1/2}$: 2.0 h and 2.7 in M / F $AUC_{0\text{-}inf}$: 801 and 761 ng h/mL in M /F Bioavailability: 58 % and 60 % (total radioactivity) & 39 % and 100 % (F9600 in plasma) for M /F <u>All groups</u>: Extensive metabolism of F9600 and limited partitioning of F9600 and its metabolites into red blood cells. Less than proportional increase in exposure with dose increase from 5 to 1000 mg/kg bw indicates non-linear kinetics in rats.

Tissue distribution **Rat** (Crl :CD9(SD), males & females, 4/sex/group) GLP OECD 417 (2010) Deviations: None **[^{14}C-Phenyl]-F9600** Batch 77874-3-19; purity 100 %; specific activity 56 mCi/mmol (202.9 µCi/mg) F9600 technical, PL14-0163; purity 99 % Study no. FMC-P4973 [redacted] **(2017b)** ***Acceptable***	Single oral low dose (5 mg/kg bw) Single oral high dose (500 mg/kg bw) Multiple oral dose (5 mg/kg bw/day; 14 days)	At T_{max} (0.5 h for single and repeated low dose group and 4 h for the single high dose group), highest tissue levels in gastrointestinal (GI) tract (~58 % of AD), carcass (up to 24 % of AD), liver (~5 % of AD) and blood (~1% of AD). No indication of preferential partition into whole blood cells. No indication of selective accumulation of bixlozone or its related metabolites in any of the tissues upon high dose administration compared to low dose. No indication of accumulation of bixlozone or its related metabolites after multiple dosing compared to single dosing. No clear differences in the distribution of bixlozone or its related metabolites between males and females.
Radioactivity concentration in plasma and bone marrow Rat (Crl :CD9(SD) 4 males GLP OECD 417 (2010) Deviations: None **[^{14}C-Phenyl]-F9600** Batch CFQ43224; purity 99.8 %; specific activity 63 mCi/mmol (228.3 µCi/mg) Study no. FMC-P7354 [redacted] **(2017f)** ***Acceptable***	Single oral low dose 500 mg/kg bw	At T_{max} (4 h), total radioactivity concentration was 153.20 µg Eq/g ± 32.46 in plasma and 49.73 µg Eq/g ± 11.94 in bone marrow. The mean bone marrow to plasma ratio was 0.33. Results provide evidence for systemic exposure, in particular exposure of rat bone marrow, at doses used in the *in vivo* rat bone marrow micronucleus assay (Section B.6.4.2).

Excretion and Metabolism Rat (Crl :CD9(SD) 4 animals/sex/group GLP OECD 417 (2010) Deviations: None of significance **[^{14}C-Phenyl]-F9600**, batch 77874-3-19; purity 100 %; specific activity 56 mCi/mmol (202.9 µCi/mg) F9600 technical, batch PL14-0163; purity 99.8 % Study no. FMC-P3887 [redacted] **(2018b)** ***Acceptable***	Single oral low dose (5 mg/kg bw) Single oral high dose (1000 mg/kg bw for M, 500 mg/kg bw for F) Multiple oral dose (5 mg/kg bw/day; 14 days)	<u>Excretion</u> M: > 90 % of the AD recovered in 7 days Major route of excretion: urine (62 – 74 % of the AD) Faeces: 21 – 34 % of the AD F: > 92 % of the AD recovered in 7 days Major route of excretion: urine (79 – 88 % of the AD) Faeces: 10 – 13 % of the AD Excretion through expired air was negligible in all dose groups in both sexes. AD recovery in tissues and carcass was minimal (day 7). Estimated oral absorption (sum of radioactivity in urine and tissues at the low dose of 5 mg/kg bw excluding faeces): **65 %** in M and 88 % in F <u>Metabolism</u> F9600 was extensively metabolised; unchanged F9600 detected at levels < 1 % of the AD in male rat urine after high dose administration only (1000 mg/kg bw). No major sex differences observed in metabolite profiles. Major metabolites identified (> 10 % of the AD in both sexes in urine after single dose at 5 mg/kg bw): 2,4-dichlorohippuric acid (12 % in M; 14.5 % in F) and 5-keto-hydrate-bixlozone (18 % in M, 24 % in F). Proposed main metabolic pathway in rats: hydroxylation leading to the formation 5-OH-F9600 and its derivatives. Other routes of metabolism included a combination of oxidation, decarboxylation and deamination followed by conjugation of oxidative derivatives.
Excretion and Metabolism (pilot study) Rat (Crl :CD9(SD) 2 animals/sex/group Not to GLP OECD 417 (2010) Deviations: N/A **[^{14}C-Carbonyl]-F9600**, batch CFQ42018; purity 99.9 %; specific activity 59 mCi/mmol (213.8 µCi/mg) F9600 technical, batch G3773:62 (PL14-0163); purity 99.5 % Study no. FMC-R3449 [redacted] **(2018a)** ***Supplementary only***	Single oral low dose (5 mg/kg bw)	<u>Excretion</u> M: 91 % of the AD recovered in 7 days. Major route of excretion: urine (67 % of the AD) Faeces: 22 % of the AD F: > 94 % of the AD recovered in 7 days Major route of excretion: urine (74 % of the AD) Faeces: 17 % of the AD Excretion was rapid. Excretion through expired air was low for both sexes. AD recovery in tissues and carcass was minimal (day 7). <u>Metabolism</u> F9600 was extensively metabolised; unchanged F9600 was not detected in urine. No major sex differences observed in metabolite profiles. Major metabolites identified (> 10 % of the AD in both sexes in urine): carbamic acid (16 % in M; 22 % in F) and 5-keto-hydrate bixlozone (26 % in M, 23 % in F).

Excretion and Metabolism Rat (Crl :CD9(SD) 4 animals/sex/group GLP OECD 417 (2010) Deviations: None of significance **[^{14}C-Carbonyl]-F9600**, batch CFQ42476; purity 99.9 %; specific activity 59 mCi/mmol (213.8 µCi/mg) F9600 technical, batch PL G3773-17; purity 99.5 % Study no. FMC-P4547 [redacted] **(2017c)** ***Acceptable***	Single oral low dose (5 mg/kg bw) Single oral high dose (1000 mg/kg bw for M, 500 mg/kg bw for F) Multiple oral dose (5 mg/kg bw/day; 14 days)	<u>Excretion</u> M: > 98 % of the AD recovered in 7 days. Major route of excretion: urine (62 % of the AD) Faeces: 34 % of the AD F: > 93 % of the AD recovered in 7 days Major route of excretion: urine (76 % of the AD) Faeces: 16 % of the AD Excretion was rapid for both sexes (> 88 % of the AD recovered after 48 h). Excretion through expired air was low for both sexes. AD recovery in tissues and carcass was minimal (day 7). <u>Metabolism</u> F9600 was extensively metabolised; unchanged F9600 was not detected in urine. No major sex differences observed in metabolite profiles. Major metabolites identified (> 10 % of the AD in both sexes in urine): carbamic acid (10 % in M; 18 % in F) and 5-keto-hydrate bixlozone (17 % in M, 23 % in F). Proposed main metabolic pathway in rats: the dimethylisoxazolidin-3-one ring moiety of bixlozone was the most susceptible site of metabolism in rats, with the phenyl ring remaining relatively well conserved. Combination of various metabolic reactions (oxidation, ring-scission, decarboxylation) lead to metabolites including oxidative ring-opened analogues and ring-cleaved analogues. The phase I metabolites, produced by various metabolic pathways, are subsequently conjugated as glucuronides in the urine.

Mass balance – bile cannulated rats Rat (Crl :CD9(SD) 5 males Not to GLP – OECD 417 (2010) Deviations: None of significance **[^{14}C-Phenyl]-F9600**, batch 77874-3-19; purity 100 %; specific activity 56 mCi/mmol (202.9 µCi/mg) F9600 technical, batch PL G3773-17; purity 99.5 % Study no. FMC-P5709 [redacted] **(2017d)** ***Not relied upon** since as IV administration was used instead of oral administration, extrapolation of the biliary excretion data to the oral route does not seem appropriate as kinetics (and in particular biliary excretion) following IV administration are likely to be different from those following oral administration.*	IV low dose (3 mg/kg bw)	Excretion M: > 90 % of the AD recovered within one day. Major route of excretion: urine (52 % of the AD) Faeces: 40 % of the AD Excretion was rapid for both sexes (> 88 % of the AD recovered after 48 h). Excretion through expired air was low for both sexes. AD recovery in tissues and carcass was minimal (day 7). Metabolism F9600 was extensively metabolised; unchanged F9600 was not detected in urine. Around 1 % of the AD was excreted through faeces, therefore gastric secretion was not significant. Predominant metabolite in bile: 5-OH-bixlozone-glucuronide (42 %) Predominant metabolite in urine: 5-OH-bixlozone-glucuronide (20 %) Proposed main metabolic pathway in rats: combination of oxidation (hydroxylation), ring-opening, and glucuronidation of oxidative products. No metabolite unique to the method employed in this study has been identified.

In vitro* comparative interspecies metabolism (first study)** Mouse, rat, dog and human hepatocytes (males, females) Not to GLP however the scientific validity of such a qualitative study design is not compromised therefore this study is acceptable for regulatory purposes. **[^{14}C-Phenyl]-F9600**, batch 77874-3-19; purity 100 %; specific activity 56 mCi/mmol (202.9 µCi/mg) **[^{14}C-Carbonyl]-F9600**, Batch CFQ42476; purity 99.9 %; specific activity 59 mCi/mmol (213.8 µCi/mg) F9600 technical, batch PL G3773-17; purity 99.5 % Study no. FMC-R4547 [redacted] **(2017e)** ***Acceptable	20 µM	[^{14}C]-bixlozone (phenyl and carbonyl) virtually completely metabolised after incubation for 4 hours in rat and dog hepatocytes. Metabolisation about 56-69 % in mouse hepatocytes and 62-86 % in human hepatocytes. Common metabolic reactions in all species: oxidation (hydroxylation) and conjugation (glucuronidation); the metabolic pathways drawn from the metabolism of bixlozone in hepatocytes are similar to those identified in rats after oral administration of [^{14}C]-bixlozone. No unique or label-specific metabolite was identified in human hepatocytes however a disproportionate production of 4-OH-Me-bixlozone was observed in human hepatocytes compared to the other species, especially the rat and dog where it was not detected. In the mouse, levels 5-8-times lower were measured. No significant sex differences or label specific metabolites were observed in human samples.

In vitro* comparative interspecies metabolism (second study)** Mixed-sex mouse, rat, dog and human hepatocytes GLP **[^{14}C-Phenyl]-F9600,** batch CFQ43508; purity 99.3 %; specific activity 61 mCi/mmol **[^{14}C-Carbonyl]-F9600, b**atch CFQ43509; purity 99.6 %; specific activity 56 mCi/mmol **F9600 technical,** batch PL14-0163; purity 99.8 %. Study no. FMC-53482 [redacted] **(2020)** ***Acceptable	20 µM	[^{14}C]-bixlozone (phenyl and carbonyl) virtually completely metabolised after incubation for 4 hours in dog hepatocytes. Metabolisation about 72-87 % of the AR in the rat, 86-92 % in mouse hepatocytes and 49-51 % in human hepatocytes. Common metabolic reactions in all species: oxidation (hydroxylation) and conjugation (glucuronidation); the metabolic pathways drawn from the metabolism of bixlozone in hepatocytes are similar to those identified in rats after oral administration of [^{14}C]-bixlozone. No unique or label-specific metabolite was identified in human hepatocytes however a disproportionate production of 4-OH-Me-bixlozone was observed in human hepatocytes compared to the other species, especially the rat.

Absorption

The extent of absorption of total radioactivity in rats administered [^{14}C-U-Phenyl]-bixlozone or [^{14}C-Carbonyl]-bixlozone orally, measured in terms of percent excretion in urine of rats, was relatively high at ≈ **60**-**70 %** in males and ≈ 80-90 % in females, with no significant differences in excretion pattern observed following low (single or repeated dosing) and high oral administration (Study # 3 Section B.6.1.1.2 & study # 6 Section B.6.1.1.5). Comparing the oral and IV AUCs obtained from the pharmacokinetics study (Study # 3 Section B.6.1.1.2), it was shown that the bioavailability of total radioactivity was higher in females (86 %) compared to males (**70 %**) following low oral dosing. A possible saturation of absorption was observed following high dose administration with lower bioavailability values retrieved at 1000 mg/kg bw in males and females (58 % and 60 % respectively) and a less than proportional internal exposure to dose in plasma and blood in both sexes, suggesting non-linear kinetics in the rat (Study # 4 Section B.6.1.1.4). No accumulation was observed in plasma following repeated dosing in both sexes.

Oral absorption was rapid following single or repeated oral low dose (5 mg/kg bw) with C_{max} reached in less than an hour post-dosing for both sexes. The rate of oral absorption was slower following oral high dose (1000 mg/kg bw), with C_{max} reached between 15-24 hours post-dosing (Study # 3 Section B.6.1.1.2).

A bile-cannulation study is available, but this has been conducted following IV dosing. As the kinetics (and in particular the biliary excretion) of bixlozone following IV administration are likely to be different from those following oral administration, HSE is of the view that the biliary and urinary excretion profiles determined from this study cannot be directly extrapolated to the oral route.

Overall, therefore, taking into account the pharmacokinetics study where an oral bioavailability value of 70% was estimated comparing the IV and oral AUCs, **HSE proposes an oral absorption value and an oral systemic availability value of 70 %.** Although there no data available to determine the absorption of bixlozone and/or its metabolites across the respiratory tract, a default inhalation absorption value of 100 % is proposed based on the extensive oral absorption observed in the rats. The dermal absorption potential of bixlozone from its representative product is addressed in the CP-B6 document.

Distribution

Available toxicokinetics data showed that the plasma $AUC_{0\text{-}inf}$ levels of unchanged bixlozone in the IV dose group were only 2-3 % of the total radioactivity, indicating extensive metabolism of bixlozone (Study # 3 Section B.6.1.1.2). Limited information on plasma concentrations of bixlozone from repeated-dose and long-

term toxicodynamic studies conducted in rats, mice and dogs confirmed there were low levels of unchanged bixlozone in blood and plasma following repeated exposure (Section B.6.3 & B.6.5).

Following oral administration bixlozone and its metabolites were widely distributed in all rat tissues by the T_{max}, and the distribution was similar between sexes (Study # 4 Section B.6.1.1.4). Among all tissues analysed, the GI tract accounted for about 60 % of the dose, followed by the carcass (up to 24 %), the liver (~5 %) and blood (~1 %). There was no indication of accumulation of radioactivity following repeated dosing.

Metabolism

In vivo, bixlozone was extensively metabolised in rats following single, high or multiple oral gavage doses, resulting in rapid and extensive excretion via urinary, bile and faecal routes; low levels, if any, of unchanged bixlozone were noted in the urine and faeces from all dose groups (Study # 6 Section B.6.1.1.5 & study # 8 Section B.6.1.1.6).

The major metabolites identified in urine in both sexes were carbamic acid (identified using [^{14}C-Carbonyl]-bixlozone), 2,4-dichlorohippuric acid (identified using [^{14}C-Phenyl]-bixlozone) and 5-keto-hydrate-bixlozone (glucuronide) (identified using [^{14}C-Phenyl]-bixlozone or [^{14}C-Carbonyl]-bixlozone). Minor metabolites identified in urine were dihydroxy-isobutyramide-bixlozone, bixlozone-dehydro-malonamide, bixlozone-dimethyl-malonamide, 4-hydroxy-methyl-bixlozone, 4-carboxy-methyl- bixlozone, 5-keto bixlozone, bixlozone-cysteine derivative and 5-hydroxy-bixlozone (glucuronide).

Based on the metabolites identified in urine and faeces, it is proposed that the dimethyl-isoxazolidin-3-one moiety of bixlozone is the most susceptible site for metabolism in rats. A combination of reactions including oxidation, reduction, decarboxylation, ring opening/cleavage, and deamination lead to extensive metabolism of bixlozone and the formation of a variety of metabolites. Several of the metabolites are subjected to conjugation with glucuronic acid for subsequent excretion in urine. The metabolites found in faeces were primarily unconjugated and could have been derived from hepatic and/or intestinal metabolism of bixlozone.

In a non-GLP comparative *in* vitro metabolism study using rat, human, mouse and dog cryopreserved hepatocytes, bixlozone was virtually completely metabolised after incubation for 4 hours in rat and dog hepatocytes, whilst the extent of bixlozone conversion to metabolites was about 56-69 % of the applied radioactivity (AR) in mouse hepatocytes and 62-86 % of the AR in human hepatocytes (Study # 10 Section B.6.1.3). No unique or label-specific metabolite was identified in human hepatocytes; however, a disproportionate production of 4-OH-Me-bixlozone was observed in human hepatocytes compared to the mouse (5-8-times higher), with none detected in the rat and dog. No significant sex differences or label specific metabolites were observed in human samples.

To address the reliability and toxicological significance of this disproportionate production of 4-OH-Me-bixlozone, the applicant submitted a second study (GLP compliant) and used hepatocytes from the same species selected for the first study; however mixed-sex hepatocytes were used instead of separated male and female hepatocytes. The findings from this study were broadly similar to the previous study and confirmed the disproportionate production of 4-OH-Me-bixlozone in human hepatocytes. Thus, the applicant provided HSE further information to evaluate the toxicological relevance of this finding. *In silico* genotoxicity comparative analysis, structural similarity analysis and a comparison of the physical-chemical properties of 4-OH-Me-bixlozone with those of the parent compound indicate the metabolite has a comparable toxicity profile to that of bixlozone. *In vivo* rat studies also showed the metabolite is rapidly eliminated in urine through oxidation to 4-COOH-Me-bixlozone and glucuronidation, suggesting that the metabolite is most likely less toxic than the parent substance. Therefore, the disproportionate production of this metabolite in human hepatocytes compared to the rat, the primary test species, is unlikely to lead to additional toxic effects beyond those already identified in the tested species as its toxicity profile is comparable to (and possibly less toxic than) that of the parent substance which has been fully tested in model experimental animals.

Lastly, due to the finding of the residues 2,2-dimethyl-3-hydroxy propionic acid (M118/1) and 2,2-dimethyl malonic acid (M132/1) in plants, and the way that these metabolites feature in the livestock metabolism studies (hen and goat; Section B.7.2), it was important to establish whether both residues had been detected in the rat metabolism studies.

In the rat metabolism studies ((Sections B.6.1.1.5 & B.6.1.1.6)), more than 40 different metabolites were observed in urine samples, with fewer metabolites retrieved in faeces samples following single or multiple oral doses ([redacted] (2018b)). Several metabolites were detected in minor to trace amounts (< 2 % of the AD) and few unknown metabolites (RP2, RP5, RP10, and RP28) present at levels ≤ 3 % of the AD were observed in the radio-chromatograms of urine samples; the structures of these metabolites could not be identified by the LC/MS method used in the study. Thus, there were some metabolites at low levels that were not identified, however

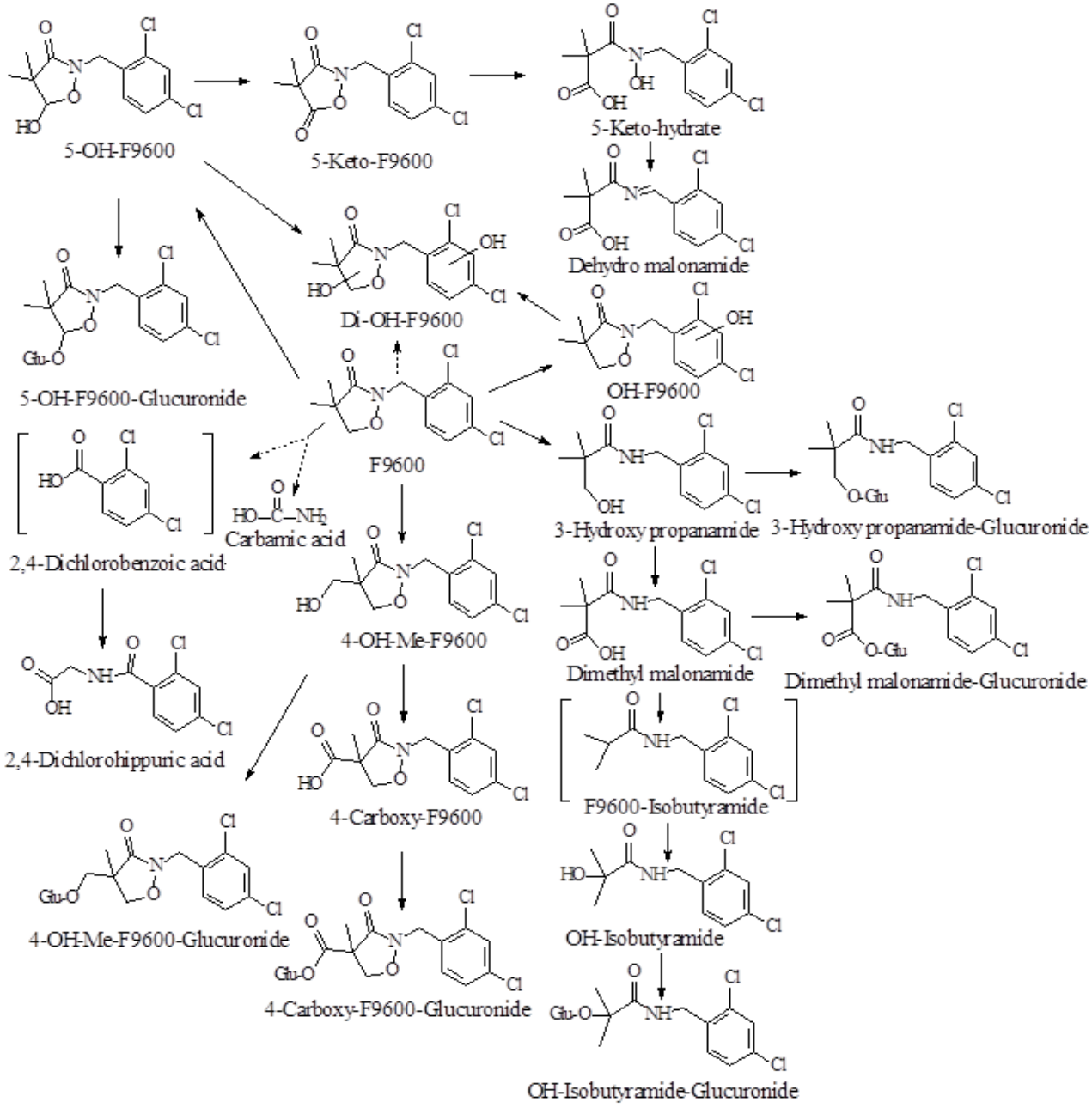
they did not actively seek M118/1 and M132/1. When comparing the metabolic pathways identified in the rat (figure B 6.1.4.1) with those identified in the goat (figure 7-7 Section B.7.2.2) and hen (figure 7-8 Section B.7.2.3), it appears that the goat and hen metabolism profiles are subsets of what is occurring in the rat. No unique metabolite paths have been identified in the goat or the hen compared to the rat. Therefore the **livestock (goat, poultry) and rat metabolism pathways are considered qualitatively similar**. Hence, it is possible that either residues M118/1 and M132/1 were present in the rat samples but were not identified, or that qualitative differences in metabolite profiles between the three species considered (goat, poultry, rat) are in play to explain the interspecies variation highlighted. Thus, although they were not identified in the rat it is likely that both metabolites could be formed in the rat.

Elimination

Excretion after a low oral dose was rapid with 83-97 % of the administered dose (AD) being excreted within 48 hours via the urine and faeces, with a higher elimination rate in females (study # 6 Section B.6.1.1.5). Although the initial rate of excretion was slightly slower in rats that received the high oral dose (69-72 % AD within 48 hours), the excretion pattern was similar between the low and high dose groups. No significant label specific differences in excretion patterns were evident. In non-bile cannulated rats, urinary excretion was relatively high (64-88 % AD with the phenyl label and 62-76 % AD with the carbonyl label), with faecal elimination accounting for 11-27 % and 16-34 % of AD for the phenyl and carbonyl label respectively. Elimination in expired air was very low with both labels. Biliary excretion was determined in bile cannulated rats following IV administration; however, HSE is of the view that the calculated value cannot be directly extrapolated to the oral route.

The proposed metabolic pathways of bixlozone in rats are presented in the figure below:

Figure B 6.1.4.1: Proposed metabolic pathways of bixlozone in the rat



Proposed residue definition for monitoring purposes in body fluids and tissues

The applicant proposed to include the metabolite 5-hydroxy-bixlozone as the only marker for monitoring purposes in body fluids and tissues on the basis that it is mostly detected as a conjugate (glucuronide) form in rats, although a portion of unconjugated metabolite may circulate as well.

The proposal was considered further by HSE. Regarding the detection of metabolite 5-hydroxy-bixlozone in rats administered bixlozone, the available ADME data showed that this metabolite was mainly found in faeces samples with very low levels in urine samples; therefore systemic exposure is unlikely to be significant. This metabolite was also mainly present in urine in its conjugated form. In addition, the *in vitro* comparative metabolism studies showed that 5-hydroxy-bixlozone (unconjugated form) is not detected in human hepatocytes (males & females) whilst levels above 10 % of the applied radioactivity were reported in rat hepatocytes in both sexes. Thus, the available data suggest that in vivo human urine / blood samples may not contain 5-hydroxy-bixlozone.

Therefore, HSE is of the opinion that 5-hydroxy-bixlozone is not a suitable marker for monitoring purposes according to the data requirements of Regulation (EU) 283/2013. In addition, the inclusion of glucuronide and sulfate conjugates in the residue definition would hinder the monitoring process because of the need for methods requiring conjugate hydrolysis. Furthermore, it is understood that an analytical method is only validated for this metabolite for animal tissues but not for body fluids; therefore the recommendations of SANCO/825/00 rev. 8.1 (16/11/2010) are not fulfilled.

HSE propose as an alternative, to include the metabolite 5-keto-hydrate-bixlozone in the residue definition, based on the fact that 5-keto-hydrate-bixlozone, a downstream metabolite of 5-hydroxy-bixlozone, is a major metabolite consistently found at high levels in its unconjugated form in rat urine samples in both sexes. Moreover, it is also consistently found in abundance in all male and female species in vitro including in human hepatocytes samples and is not observed in the *in vivo* and *in vitro* samples in its conjugated form. The applicant agreed with the HSE proposal.

Therefore, the metabolite 5-keto-hydrate-bixlozone is considered to be a relevant analyte identified in the toxicological database and is suitable as a typical marker to be included in the residue definition for the monitoring of body fluids and tissues.

A validated analytical method for analysis of bixlozone (parent) and the marker metabolite 5-keto-hydrate-bixlozone in body fluids (plasma and urine) and tissues (liver) is available. Therefore, the data requirements of Regulation (EU) 283/2013 have been met.

B.6.2. ACUTE TOXICITY

The acute toxicity of bixlozone has been investigated *in vivo* in rats via the oral (OECD 425), dermal (OECD 402) and inhalation (OECD 403) routes.

In vivo skin (OECD 404) and eye (OECD 405) irritation studies have been submitted to HSE with no justification for their use provided by the applicant. The first *in vitro* validated tests addressing these two toxicology endpoints, skin (OECD guideline 439) and eye (OECD guidelines 437 and 438) irritation and/or corrosion potential have been available since 2010 and 2009, respectively. Following a request for further information the applicant confirmed that vertebrate studies were undertaken for the purposes of global submission as not all countries accept *in vitro* assays *in lieu* of *in vivo* data, and that no *in vitro* skin and eye corrosion/irritation studies were considered prior to the conduct of the vertebrate studies. As there were no indications of corrosion or pain reactions observed in the acute dermal toxicity study conducted in the rat, and as the pH of the test substance dissolved in water was measured by the conducting laboratory to be 4.5, HSE considered that no conditions existed which would have prevented the conduct of *in vitro* studies. Since Article 62 of Regulation (EC) No 1107/2009 makes it clear that testing on vertebrate animals shall be undertaken only where no other methods are available, *in vitro* skin and eye irritation / corrosion studies should have been conducted prior commissioning any *in vivo* studies. Thus, the justification was not considered acceptable. The applicant subsequently submitted an *in vitro* skin irritation test (OECD 439) and an eye irritation / corrosion test (OECD 492), and these are presented in this Section of the DAR.

The skin sensitisation potential of bixlozone was investigated in a mouse local lymph node assay (LLNA; OECD 429). The phototoxic potential of bixlozone did not need to be addressed as the UV absorbance criteria were not met.

B.6.2.1. Oral

The acute oral toxicity of bixlozone was investigated in rats using the up-and-down procedure as described in OECD TG 425 (2008).

Study	F9600 Technical: Acute Oral Toxicity – Up-and-Down Procedure in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014a)
Date performed	Dec 2013
Test facility	[redacted]
Report reference	Study no. 37755 - FMC reference 2013TOX-ISX0999
Guideline(s)	OECD 425 (2008)
Deviations from the guideline	None
GLP	Yes
Test material	F9600 technical, batch PL13-0203 Purity: 98.5 %
Study acceptable	Yes

Material, Methods and Results

The acute oral toxicity of bixlozone was investigated in five fasted female Sprague-Dawley albino rats in accordance with the up-and-down procedure (OECD TG 425). Prior to the start of the test, the LD_{50} of bixlozone was estimated to be 1369 mg/kg bw (no further information provided). Based on this estimation, an initial animal was given a single gavage dose of 430 mg/kg bw (the next dose below the estimated LD_{50}). This animal survived and did not display any signs of toxicity. Therefore, a second animal was dosed with 1370 mg/kg bw of the test substance; the survival of this rat led to the dosing of further three animals at the maximum recommended dose of 2000 mg/kg bw. A method of analysis for the test substance concentration in the gavage solution is not required for this study.

All three animals, treated with the maximum dose, survived the 14-day observation period; the only signs of toxicity were observed in two females on the first day of treatment: hypoactivity, irregular respiration (3-5 hours post dose) and decreased defaecation volume. These signs did not indicate a specific toxic effect and had fully reversed by day two; all three animals appeared active and healthy for the remainder of the 14-day observation period. All animals gained weight during the study and no gross abnormalities were noted upon necropsy. The survival of three animals at the top-dose of 2000 mg/kg bw concluded the study.

Conclusion

The acute oral LD_{50} of bixlozone in female rats was shown to be greater than the highest dose tested of 2000 mg/kg bw, which led to conclusion that bixlozone is of low acute toxicity via the oral route and does not meet the criteria for classification in accordance with Regulation (EC) N°1272/2008.

[redacted] (2014a)

Table B 6.2.1.1: Summary of the acute oral toxicity study of bixlozone

Method, Species, Guideline	Test substance, doses	LD_{50}	Effects
Acute oral toxicity study, up-and-down procedure, gavage Rats, SD albino, five females Vehicle: 0.5 % aqueous solution of CMC in 5 % Tween-80 Observation period: 14 days Guideline: OECD 425 (2008) Deviations: none GLP [redacted] (2014a) **Acceptable**	F9600 technical, batch PL13-0203 Purity: 98.5 % Doses: 430 (n=1), 1370 (n=1) & 2000 mg/kg bw (n=3)	**> 2000 mg/kg bw**	There were no deaths or clinical signs of toxicity at any dose level. Effects observed at the maximum dose 2000 mg/kg bw: Day 1: hypoactivity , irregular respiration (3-5 hours) and ↓ defaecation in 2/3 (recovered by day 2)

B.6.2.2. Dermal

The acute dermal toxicity of bixlozone was investigated in rats according to OECD TG 402 (1987).

Study	F9600 Technical: Acute Dermal Toxicity Study in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014b)
Date performed	Dec 2013
Test facility	[redacted]
Report reference	Study no. 37756, FMC reference 2013TOX-ISX0998
Guideline(s)	OECD 402 (1987)
Deviations from the guideline	Application site constituted less than 10 % surface area (approx. 8%) owing to the small quantity of test material applied; however, signs of dermal irritation were clearly minimal in the study. The Guidance on the Application of the CLP Criteria (version 5.0 July 2017) states in Section 3.1.2.3.2. for the evaluation of non-human data for acute toxicity that "if a substance is not acutely toxic by the oral route it can also be assumed that it is not acutely toxic by the dermal route." Since bixlozone is not acutely toxic via the oral route, this deviation from the OECD guideline is not expected to have a significant impact on the outcome of the present study.
GLP	Yes
Test material	Bixlozone technical, batch PL13-0203 Purity: 98.5 %
Study acceptable	Yes

Material and Methods

In an acute dermal toxicity study the shorn skin of five/sex Sprague-Dawley albino rats was exposed to a 2000 mg/kg bw dose of bixlozone technical (batch PL13-0203; purity 98.5 %). The dose was formulated as a 70 % w/w paste with distilled water and applied to the skin with a semi-occlusive dressing (taped onto an area of approximately 3.5 cm x 2.5 cm). It is noted that this application site constituted approximately 8 % of the total body surface area (less than the 10 % recommended in the test guideline). The applicant reported that this was

the maximum area that could be covered owing to the small quantity of test material applied. Since signs of dermal irritation were minimal in the study and bixlozone was not found to be acutely toxic via the oral route (LD_{50} > 2000 mg/kg bw), HSE considers that this deviation is not expected to have had a significant impact on the outcome of the present study. A method of analysis for the test substance concentration is not required for this study.

An exposure period of 24 hours was allowed prior to removal of the pad and cleansing of the exposed skin area. A 14 day observation period then followed in which mortality, signs of toxicity and behavioural changes were monitored daily. Body weights were recorded prior to administration of the test substance and then weekly thereafter (days 7 and 14). Gross necropsies were performed on all animals.

Results

There were no deaths or clinical signs of toxicity; all animals gained weight during the study and no gross abnormalities were noted upon necropsy. Dermal irritation (erythema) was noted at the dose site of two out of five males only on day one, which had fully reversed by day 2. No gross abnormalities were noted upon necropsy.

Conclusion

Under the conditions of this study the acute dermal LD_{50} of bixlozone was shown to be greater than the highest dose tested of 2000 mg/kg bw, and in accordance with Regulation (EC) N°1272/2008 the criteria for classification have not been met.

[redacted] (2014b)

Table B 6.1.3.3: Summary of the acute dermal toxicity study of bixlozone

Method, Species, Guideline	**Test substance, doses**	**LD_{50}**	**Effects**
Acute dermal toxicity study Rats, SD albino, 5/sex Guideline: OECD 402 (1998) Deviations (not significant): Application site constituted 8 % of the surface area (less than required 10 %) GLP [redacted] (2014b) **Acceptable**	F9600 technical, batch PL13-0203 Purity: 98.5 % Dose: 2000 mg/kg Moistened with distilled water to a dry paste (70 % w/w) Exposure period: 24 hours Observation period: 14 days	**> 2000 mg/kg bw**	Dermal irritation (erythema) at dose site in two out of five males only on day one (fully reversible by day two).

B.6.2.3. Inhalation

The acute inhalation toxicity of bixlozone was investigated in rats using a nose-only exposure system according to OECD TG 403 (2009).

Study	F9600 Technical: Acute Inhalation Toxicity in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014c)
Date performed	Jan 2014
Test facility	[redacted]
Report reference	Study no. 37947, FMC reference 2013TOX-ISX1000
Guideline(s)	OECD 403 (2009)
Deviations from the guideline	Yes - A validated method of analysis is not available for this study. The tested concentration was verified gravimetrically, by weighing the material present on the Whatman filter papers that are 37 mm in diameter and have a pore size of 1 µm. This

	method is generally considered fit for purpose for this type of study. This limitation does not impact the validity of the study.
GLP	Yes
Test material	F9600 technical, batch PL13-0385 Purity: 99.2 %
Study acceptable	Yes

Material and methods

In an acute inhalation toxicity study, groups of five/sex Sprague-Dawley albino rats were exposed for four hours (nose-only) to bixlozone technical supplied as aerosolised dust (batch PL13-0385, purity 99.2 %).

The test substance was milled and sieved before being packed and compressed into a dust container. The prepared dust was then aerosolised using a dust generator and fed directly into the chamber through a dust outlet assembly. Approximately 30.0 litres per minute (Lpm) of filtered generator air was supplied by an air compressor to the dust generator. An additional 6.0 Lpm of filtered mixing air from the same air compressor was introduced into the chamber to help uniformly distribute the test atmosphere by creating a vortex at the chamber inlet.

The tested concentration was 2.11 mg/L and the average mass median aerodynamic diameter (MMAD) was 2.84 µm. A validated method of analysis is not available for this study. However, the tested concentration was verified gravimetrically, by weighing the material present on the Whatman filter papers that are 37 mm in diameter and have a pore size of 1 um. This method is generally considered fit for purpose for this type of study.

After exposure, the animals were observed for a 14-day period in which they were examined for signs of toxicity and behavioural changes immediately following their removal from the chamber and at least once daily thereafter. Body weights were recorded prior to exposure and subsequently on days one, three, seven and 14.

The concentration tested in this study (2.11 mg/L) is lower than the maximum concentration of 5 mg/L recommended in the OECD Guideline 403. Thus, the concentration tested did not cover all of the four hazard categories set in Annex I: 3.1.2.1. of the guidance on the Application of the CLP criteria for this endpoint (i.e., up to 5 mg/L). However, the testing conditions achieved in this study were conform with the recommendations of the OECD Guideline 403 regarding the testing of aerosols. Paragraph 30 of the OECD guideline specifies that it can be technically challenging to generate limit concentrations for aerosols. It also states that when testing aerosols, the primary goal should be to achieve a respirable particle size (MMAD of 1-4 µm), which is possible with most test articles at a concentration of 2 mg/L, which is the case for this study. Lastly, paragraph 30 also highlights that aerosol testing at greater than 2 mg/L should only be attempted if a respirable particle size can be achieved. These recommendations are also detailed in Annex I Section 3.1.2.3.2 of the CLP guidance, in that for dusts and mists, a particle size range of 1-4 µm, corresponding to a maximum concentration of about 2 mg/l, would be tested in rats to achieve applicability of the animal experiment conditions to human exposure.

Therefore, HSE concludes that the study tested bixlozone technical at the maximum achievable concentration recommended for aerosols by the OECD guideline and the CLP guidance. Therefore, the study is valid for the purpose of classification.

Results

There were no deaths; all rats exhibited irregular respiration following exposure but fully recovered by day 3. Although there were minor individual body-weight losses observed at various weighting time-points, overall, by the end of the study, the animals had gained the expected amount of weight. The sporadic weight losses observed were therefore not of toxicological significance. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period. Thus, the 4-hr-LC_{50} was determined to be > 2.11 mg/L.

Conclusion

Under the conditions of this study the acute inhalation 4hr-LC_{50} (single exposure) of bixlozone (aerosol) was greater than the concentration tested of 2.11 mg/L. Overall, bixlozone does not meet the classification criteria for acute inhalation toxicity in accordance with Regulation N°1272/2008, noting that the study tested the aerosolised substance up to the maximum attainable concentration of 2.11 mg/L.

[redacted] (2014c)

Table B 6.1.3.3: Summary of the acute inhalation toxicity study with bixlozone

Method, Species, Guideline *Acceptability*	**Test substance, doses**	**LC_{50}**	**Effects**
Acute inhalation toxicity study, nose-only Rats, SD albino, males & females, five/sex Guideline: OECD 403 (2009) Deviations: Yes. An adequate method of analysis for the concentration tested is not available but the method was concluded fit for purpose. Does not impact the validity of the study. GLP [redacted] (2014c) **Acceptable**	Bixlozone technical aerosol, batch PL13-0385 Purity: 99.2 % 2.11 mg/L (maximum attainable concentration) MMAD: 2.84 µm Exposure: 4 hr	> **2.11 mg/l**	There were no deaths Clinical signs: irregular respiration in all animals following exposure (full recovery by day three)

B.6.2.4. Skin irritation

The skin irritation potential of bixlozone was investigated in an *in vitro* skin irritation test (OECD 439; bottom-up approach) and an *in vivo* study (OECD 404).

B.6.2.4.1. *In vitro skin irritation test*

A GLP and OECD compliant *in vitro* skin irritation test using the Epiderm™ skin model study (capable of distinguishing between no classification and classification) is available and was performed in response to queries from HSE regarding the provision of vertebrate data.

Study	F9600 Technical: *In vitro* skin irritation test (SIT) using the Epiderm™ skin model
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	Costin, GE. (2018)
Date performed	April 2018
Test facility	Institute for In Vitro Sciences, Inc. USA
Report reference	Study no. 18AC10.050082
Guideline(s)	OECD 439 (2015) – Reconstructed human epidermis (RhE) test
Deviations from the guideline	There were no deviations of significance
GLP	Yes
Test material	F9600 technical batch PL14-0049 Purity: 95.9 %
Study acceptable	Yes

Material and Methods

After on overnight conditioning and pre-incubation period, triplicate tissue samples designated for treatment with 25 mg of bixlozone technical (powder) were moistened with 25 µL sterile Ca^{++}/Mg^{++} Free Dulbecco's Phosphate Buffered Saline (CMF-DPBS) to improve contact between the test substance and the tissue surface. A method of analysis for the test substance concentration is not required for this study.

The positive control Sodium Dodecyl Sulphate (SDS, 5 %) and negative control CMF-DPBS were tested concurrently and applied to tissue samples in triplicate. All samples were incubated for 60 minutes and subsequently thoroughly rinsed, blotted, and transferred to fresh medium. After a 24-hour post-exposure

incubation, tissue samples were supplied with fresh medium and incubated for further 18 hours for a total of 42-hour post exposure period.

Cell viability was assessed using the 3- [4,5 – dimethylthaizol-2-yl] – 2,5 – diphenyltetrazolium bromide (MTT) assay where the blue formazan salt formed in the test item-treated tissue was measured relative to the negative controls. Bixlozone technical was also tested in a preliminary experiment to identify its potential in being a direct MTT reducer or for colour interference in contact with water or isopropanol.

The assay was considered acceptable if the following laboratory criteria, based on OECD Guideline 439, were met:

- the positive control (5 % SDS) resulted in a mean tissue viability ≤ 20 %;
- the mean OD_{570} value of the negative control tissues was ≥ 0.8 and < 2.8;
- the standard deviation (SD) calculated from the individual percent tissue viabilities of the three identically treated replicates of the negative or positive control was < 18 %.

Results

Preliminary test article compatibility assays

Bixlozone technical was not observed to directly reduce MTT in absence of viable cells or to have probable photometric MTT interference (OD_{570} value at 0.003).

Main skin irritation test

According to the OECD Guideline 439 criteria, a test article is predicted to be an irritant/corrosive (GHS Category 1 or 2) when the mean relative viability of the three treated tissues is less than or equal to 50 % of the mean viability of the negative control. The mean viability of bixlozone technical was 109 %. Both, the positive control SDS (viability of 2.6 %; SD < 18 %) and the negative control CMF-DPBS (mean OD_{570} at 1.922; SD < 18 %) met the laboratory criteria for a valid assay.

The results of the main skin irritation test are presented in the table below:

Table B 6.2.4.1: Results of the *in vitro* skin irritation test

Test item		Concentration	Mean Viability (%) ± SD	Skin Irritation Prediction
F9600	F9600 Technical, powder	Neat 25 mg	109.1 ± 4.59	Non-Irritant
Positive Control	SDS	5 % w/v	2.59 ± 0.53	Irritant
Negative Control	CMF-DPBS	-	100.0 ± 3.45	Non-Irritant

Conclusion

Under the conditions specified in this study, bixlozone technical was predicted to be non-irritant to the skin and would not require classification and labelling for skin irritation according to Regulation (EC) 1272/2008.

Costin, GE. (2018)

B.6.2.4.2. *In vivo skin irritation test*

The skin irritating potential of bixlozone has also been investigated *in vivo* in rabbits.

Study	F9600 Technical: Primary Skin Irritation Study in Rabbits
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014d)
Date performed	Nov 2013
Test facility	[redacted]
Report reference	Study no. 37758, FMC reference 2013TOX-ISX0995
Guideline(s)	OECD 404 (2002)
Deviations from the guideline	None
GLP	Yes
Test material	F9600 technical, batch PL13-0203 Purity: 98.5 %

Study acceptable	No. The study should not have been conducted as the available *in vitro* test is sufficient and thus the results from the study are not relied upon.

Material and methods

Bixlozone technical (batch PL13-0203, purity 98.5 %) was applied to the clipped dorsal skin of three female rabbits. A moist paste (70 % w/w volume) was achieved by mixing 0.5 g of the test substance with 0.21 g of distilled water, which was then applied under a semi-occlusive dressing for a 4-hour exposure period. A method of analysis for the test substance concentration is not required for this study.

The animals were observed for signs of toxicity and behavioural changes once daily until the conclusion of the study (72 hours). Body weights were recorded prior to dosing and at the conclusion of the study.

The dose sites were scored for erythema and oedema at 30-60 minutes and then at 24, 48 and 72 hours post-exposure. As required by the CLP criteria, the scores taken for 30-60 minutes were not included in the calculation for the mean scores, which was based on scores over 24, 48 and 72 hours for each animal.

Results

All animals appeared active and healthy and gained body weight during the study. There were no deaths or signs of toxicity reported. Apart from the mild dermal irritation described below, there were no other signs of gross toxicity, adverse pharmacologic effects, or abnormal animal behaviour.

There was no oedema observed at any treated site during this study. Within one hour of patch removal, all three treated skin sites exhibited slight erythema. The mean scores for erythema for each rabbit were 0, 0.67 and 0.33 and the mean scores for oedema were 0 for each rabbit. All observations had fully reversed by 72 hours.

Table B 6.2.4.2: Dermal irritation results

Animal No.	Sex	Individual skin irritation scores (erythema / oedema)				Mean score*
		Time After Patch Removal				
		30-60 min	24 hrs	48 hrs	72 hrs	
3501	F	*1/0*	0/0	0/0	0/0	0/0
3502	F	*1/0*	1/0	1/0	0/0	0.67/0
3503	F	*1/0*	1/0	0/0	0/0	0.33/0

*Grading of erythema/oedema formation: 0 (no formation); 1 (very slight formation); 2 (well defined erythema/slight oedema); 3 (moderate to severe); 4 (severe formation)

Conclusion

Bixlozone was mildly irritating to the skin (erythema). This effect had fully reversed by 72 hours. The mean scores for each rabbit were not sufficient for classification as a skin irritant according to the CLP criteria. Bixlozone was not found to be a skin irritant under the conditions of this study. No classification for skin irritation is required according to Regulation (EC) N°1272/2008.

[redacted] (2014d)

Overall conclusion on skin irritation

Two GLP and OECD compliant studies were conducted to specifically investigate the skin irritating potential of bixlozone: an *in vitro* skin irritation test (SIT) using the Epiderm™ skin model (OECD Guideline 439) and an *in vivo* study in rabbits (OECD Guideline 404).

In vitro, bixlozone was not predicted to be a skin irritant. The RhE-based test method used for this study is able to distinguish between skin irritant and not skin irritant chemicals when a bottom-up approach strategy is used and can thus serve as a stand-alone skin irritation method for non-corrosive substances (OECD new guidance document on an Integrated Approach on Testing and Assessment (IATA) for skin corrosion and irritation (ENV/JM/MONO(2014)19)).

The bottom-up approach should be followed when all available collected information indicate there is a high a-priori probability of the chemical being not a skin-irritant. It cannot be excluded that in some situations a skin corrosive chemical is correctly identified as corrosive in the *in vitro* RhE-based skin corrosion test methods but

found to be non-irritant in the *in vitro* RhE-based skin irritation test methods (ENV/JM/MONO(2014)19 paragraph 93).

Other toxicity data of dermal exposure are available for bixlozone: an acute dermal toxicity study (OECD Guideline 402, [redacted] (2014b)) and a 21-day repeated-dose toxicity study (OECD Guideline 410, [redacted] (2016)), both conducted in rats. Although it is recognised that the dosing design of these studies significantly differs from a local acute skin irritation / corrosion study, the local effects reported from these studies may be helpful for the weight of evidence assessment of the probability of bixlozone to be irritant to the skin. In the acute dermal toxicity study, dermal irritation (erythema) was noted at the dose site of two out of five males only on day one, and fully reversed by day two. It was noted that the application site constituted less than the recommended 10 % surface area owing to the small quantity of test material applied; however, the signs of dermal irritation were clearly minimal. Regarding the 21-day dermal toxicity study, there were no test substance-related dermal observations since all sites were scored as unremarkable at all observation periods. Thus, according to the available dermal data, bixlozone is not expected to be corrosive to the skin but may exert mild skin-irritation. Therefore, the choice of the SIT test (OECD 439) was appropriate.

In the *in vivo* skin irritation/corrosion study conducted by [redacted] (2014d) bixlozone was found to be mildly irritating to the skin (erythema) with local effects reversed by 72 hours. The mean scores for each rabbit were not sufficient for classification as a skin irritant according to the CLP criteria. The results from this study support the results from the *in vitro* study and other available data, i.e. that bixlozone is not irritant to the skin. However, since the validated *in vitro* test method (OECD Guideline 439) has been available since 2010 and can be used as a stand-alone skin irritation method to determine the skin irritation classification of test substances, HSE considers that the *in vitro* study should have been conducted prior to considering any further *in vivo* testing. Therefore, the results from the *in vivo* study conducted by [redacted] (2014d) in rabbits will not be relied upon to conclude on the classification of bixlozone for skin irritation/corrosion.

Overall bixlozone was found to be not irritating to the skin and does not require classification according to Regulation (EC) 1272/2008.

Table B 6.2.4.3: Summary of skin irritation studies conducted with bixlozone

Method, Species, Guideline	Test substance, doses	Results
In vitro skin irritation test (SIT) EpidermTM skin model (RhE-based test method) Guideline: OECD 439 (2015) Deviations: none GLP Costin, GE. (2018) **Acceptable**	F9600 technical batch PL14-0049 Purity: 95.9 % Dose: 25 mg (powder)	Mean viability of bixlozone technical = 109 %. Skin Irritation Prediction = Not a skin irritant. No classification
Primary skin irritation study Rabbits, New-Zealand albino, females, 3 animals Guideline: OECD 404 (2002) Deviations: none GLP [redacted] (2014d) **Study not relied upon**	F9600 technical, batch PL13-0203 Purity: 98.5 % Dose: 0.5g (70 % w/w paste) Vehicle: Distilled water	Mean scores over 24, 48 & 72 hours for each animal: 0, 0.67 & 0.33 (erythema) 0, 0 & 0 (oedema) Mildly irritating but insufficient for classification

B.6.2.5. Eye irritation

The eye irritation potential of bixlozone was investigated in an *in vitro* eye irritation test (OECD 492 – bottom-up approach) capable of determining whether no classification is appropriate, and an *in vivo* study (OECD 405).

B.6.2.5.1. *In vitro eye irritation / corrosion test*

A GLP and OECD compliant *in vitro* Epiocular™ eye irritation test is available and was performed in response to queries from HSE regarding the provision of vertebrate data.

Study	F9600 Technical: Epiocular™ eye irritation test (EIT) for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	Wilt, N. (2018)
Date performed	April 2018
Test facility	Institute for In Vitro Sciences, Inc.
Report reference	Study no. 18AC10.015091
Guideline(s)	OECD 492 (2017) – Reconstructed human cornea-like epithelium (RhCE) test
Deviations from the guideline	There were no deviations of significance
GLP	Yes
Test material	F9600 technical batch PL14-0049 Purity: 95.9 %
Study acceptable	Yes, but only supplementary

Material and Methods

After an overnight conditioning and pre-incubation period, tissue model inserts consisting of stratified human keratinocytes were exposed to 50 mg of solid bixlozone technical for 6 hours in duplicate. The positive control, methyl acetate: CAS 79-20-9 (neat), and negative control, CMF-DPBS, were tested concurrently and applied to tissues also in duplicate. Tissue samples were subsequently thoroughly rinsed, blotted, and transferred to fresh medium for 18 hours. A method of analysis for the test substance concentration is not required for this study.

Cell viability was assessed using the 3- [4,5 – dimethylthaizol-2-yl] – 2,5 – diphenyltetrazolium bromide (MTT) assay where the blue formazan salt formed in the test item-treated tissue was measured relative to the negative controls. Bixlozone technical was also tested in a preliminary experiment to identify its potential in being a direct MTT reducer or for colour interference in contact with water or isopropanol.

The assay was accepted if the following laboratory criteria, based on OECD TG 492, were met: the negative control OD was > 0.8 and < 2.5, and the mean relative viability of the positive control was ≤ 50 % .

Results

Preliminary test article compatibility assays

Bixlozone technical was not observed to directly reduce MTT in absence of viable cells. Since the isopropanol samples for bixlozone technical had a mean corrected OD_{550} value of 0.005, bixlozone was not considered to have probable photometric MTT interference.

Definitive eye irritation test

The test substance is predicted to have eye irritation/corrosion potential if the relative viability is less than or equal to 60 %. If the relative viability is greater than 60 %, then the test substance is not an eye irritant/corrosive and does not require classification for ocular irritation (GHS No Category). Both, the positive control, methyl acetate (viability at 13.4 %) and the negative control, CMF-DPBS (mean OD_{550} at 1.53) met the laboratory criteria for a valid assay. Moreover, the difference in viability between two tissue replicates were less than 20 % for all test items, as required by the OECD Guideline acceptance criteria.

The results of the main eye irritation test are presented in the table below:

Table B 6.2.5.1: *In vitro* Eye irritation results

Test items		Concentration	Exposure Time	Mean Viability (%)	Ocular Irritation Prediction
F9600	F9600 Technical	Neat	6 hours	19.4	Irritant
Positive Control	Methyl acetate	Neat		13.4	Irritant
Negative Control	CMF-DPBS	-		100	Non-irritant

Conclusion

Under the conditions specified in this GLP and OECD compliant *in vitro* eye irritation study using the EpiOcular™ test system, bixlozone is predicted to be an ocular irritant (potentially inducing eye irritation or serious eye damage) with a cell viability of 19.4 %.

Wilt, N. (2018)

B.6.2.5.2. *In vivo eye irritation / corrosion test*

The eye irritating potential of bixlozone was investigated *in vivo* in rabbits.

Study	F9600 Technical: Primary Eye Irritation in Rabbits.
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014e)
Date performed	Nov 2013
Test facility	[redacted]
Report reference	Study no. 37757, FMC Reference 2013TOX-ISX0996
Guideline(s)	OECD 405 (2012)
Deviations from the guideline	None
GLP	Yes
Test material	F9600 technical, batch PL13-0203 Purity: 98.5 %
Study acceptable	Yes

Material and Methods

Bixlozone technical (batch PL13-0203, purity 98.5 %) was administered as a single instillation of 0.1 ml of soluble 20 % w/w mixture in a 0.5 % solution of CMC in 5 % Tween-80 and acetone/olive oil (AOO; 4:1 v/v) into the right eye of three New Zealand albino rabbits; the untreated left eye served as the negative control. Ocular irritation was evaluated using a high-intensity white light at 1, 24, 48 and 72 hours post instillation (with an additional fluorescein dye procedure at 24 hours to evaluate corneal damage).

Results

There were no deaths or signs of toxicity and the animals gained the expected amount of weight by the end of the study period.

No corneal opacity or iritis was observed in any treated eye. Conjunctival redness and chemosis were noted in all treated eyes to some extent, but only at one hour post-installation and this did not affect the calculation of the mean scores (calculated for 24-72 hours) which were all zero.

Table B 6.2.5.2: Eye irritation results

Rabbit	Incidence of Positive Effects	Time post-instillation				Mean score (24-72 hours)
		1h	24h	48h	72h	
3401 (female)	Corneal Opacity	0	0	0	0	0.00
	Iritis	0	0	0	0	0.00
	Conjunctival redness	1	0	0	0	0.00

	Conjunctival chemosis	1	0	0	0	0.00
3402 (female)	Corneal Opacity	0	0	0	0	0.00
	Iritis	0	0	0	0	0.00
	Conjunctival redness	1	0	0	0	0.00
	Conjunctival chemosis	1	0	0	0	0.00
3403 (female)	Corneal Opacity	0	0	0	0	0.00
	Iritis	0	0	0	0	0.00
	Conjunctival redness	2	0	0	0	0.00
	Conjunctival chemosis	1	0	0	0	0.00

Conclusion

Bixlozone caused minimal irritation to the eye one hour after treatment, which had fully recovered by 24 hours. Mean scores for each animal for corneal opacity, iritis and conjunctival redness and chemosis over 24, 48 and 72 hours were 0, 0 and 0. Hence, bixlozone does not meet the criteria for classification as an eye irritant under the conditions of this study and according to Regulation (EC) N°1272/2008.

[redacted] (2014e)

Overall conclusion on eye irritation

Two GLP and OECD compliant studies were conducted to investigate the eye irritating potential of bixlozone: an *in vitro* Epiocular™ eye irritation test (OECD Guideline 492), aiming to identify test items not classified for eye irritation / damage, and an *in vivo* study in rabbits. *In vitro*, bixlozone was predicted to be an ocular irritant/eye damaging substance.

According to the OECD Guidance Document on an Integrated Approach on Testing and Assessment (IATA) for Serious Eye Damage and Eye Irritation (ENV/JM/MONO(2017)15) on the use of the bottom-up approach to identify test items not classified for eye irritation / damage, further testing may be considered in case a positive result is obtained with an *in vitro* test, such as the OECD guideline 492, as this test produces a high rate (37 %) of false positives. An *in vivo* study is available for bixlozone and showed that bixlozone caused minimal irritation to the eye one hour after treatment which had fully recovered at 24 hours. The mean scores for each animal for corneal opacity, iritis and conjunctival redness and chemosis over 24, 48 and 72 hours were 0 and did not meet the criteria for classification as an eye irritant under the conditions of this study.

Overall, taking into consideration all the information available on the eye irritating potential of bixlozone, HSE concludes that bixlozone is not an eye irritant according to Regulation (EC) N°1272/2008. The positive result obtained in the *in vitro* test is considered to represent a false positive and this is confirmed by the negative result of the *in vivo* test, which is fully relied upon.

Table B 6.2.5.3: Summary of the eye irritation study of bixlozone

Method, Species, guideline	Test substance, doses	Results
In vitro eye irritation study Epiocular™ eye model ((RhE-based test method) Guideline: OECD 492 (2017) Deviations: none GLP Wilt, N. (2018) **Supplementary**	F9600 technical batch PL14-0049 Purity: 95.9 % Dose: 25 mg (powder)	Mean viability of bixlozone Technical = 19.4 %. Eye Irritation Prediction = eye irritant/damaging.

Method, Species, guideline	Test substance, doses	Results
Primary eye irritation study Rabbits, New Zealand albino, females, 3 animals Guideline: OECD 405 (2012) Deviations: none GLP [redacted] (2014e) **Acceptable**	F9600 technical, batch PL13-0203 Purity: 98.5 % Dose: 0.08 g from a soluble 20 % w/w mixture (0.1 ml) Vehicle: 0.5 % solution of CMC in 5 % Tween-80 and acetone/olive oil (AOO; 4:1 v/v)	Mean scores at 24, 48 and 72 hours for each animal Corneal opacity: 0,0,0 Iritis: 0,0,0 Conjunctival redness: 0,0,0 Conjunctival chemosis: 0,0,0 Not classified

B.6.2.6. Skin sensitisation

The skin sensitising potential of bixlozone was investigated in a mouse local lymph node assay (LLNA).

Study	F9600 Technical: Local Lymph Node Assay (LLNA) in Mice
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014f)
Date performed	Dec 2013
Test facility	[redacted]
Report reference	Study no. 37759, FMC Reference 2013TOX-ISX0997
Guideline(s)	OECD 429 (2010)
Deviations from the guideline	None
GLP	Yes
Test material	bixlozone technical, batch PL13-0203 Purity: 98.5 %
Study acceptable	Yes

Material and Methods

A mouse LLNA assay was available which assessed the skin sensitising potential of bixlozone. A preliminary toxicity test was conducted in order to determine the highest possible concentration for the main test that would not cause excessive toxicity or local effects (2 animals/group). In the main test doses of 5 %, 10 % and 25 % (25 µl each) in vehicle acetone/olive oil (4:1 v/v) were spread over the dorsum of both ears of 5 mice/group for 3 consecutive days (days 4, 5 and 6 were treatment-free). A negative (vehicle) control was included (5 animals) and the moderate contact sensitiser compound alpha-hexyl-cinnamaldehyde (HCA) (97.9 % purity) served as the positive control (5 animals). Scoring for erythema and oedema was carried out prior to each application and subsequently on days 1, 2, 3 and 6. On day six 20 µCi of ^{3}H-methyl thymidine in PBS was injected into the tail vein of each mouse and animals were sacrificed 5 hours later. Body weights for each animal were recorded prior to test substance administration and then prior to sacrifice on day 6.

Lymph nodes were evaluated for individual animals (measured by the extent of ^{3}H-methyl thymidine incorporation) determined by B-scintillation counting and expressed as disintegrations per minute minus background (dpm). The mean dpm values for each dose-group were used to calculate the stimulation index (SI) value.

Results

Preliminary study

Test substance concentrations of 5 %, 10 %, and 25 % were tested in the preliminary study to determine the highest achievable level that avoids overt systemic toxicity and excessive local irritation; solutions in excess of 25 % were considered to be too viscous for application. No irritation was noted at any of these doses. Therefore, based on a combination of knowledge of the test substance (toxicity, solubility, irritancy and

viscosity) along with the findings of this preliminary toxicity study, animals were assigned to the following dose groups for the main study, with the 25% concentration being considered the maximum attainable concentration:

Group	Test material	Concentration (%)
1	Vehicle (negative control)	0
2	HCA (positive control)	25
3	bixlozone	5
4		10
5		25

Main study

There were no deaths. Seven treated mice (4 in the group treated with 5 % bixlozone and 3 in the group treated with 10 % bixlozone) and 2 mice in the positive control group lost weight or failed to gain weight; otherwise all animals were observed as active and healthy throughout the study. These effects were not considered to be treatment-related.

There was no dermal irritation observed in any of the animals in the vehicle control or bixlozone groups. In the positive control group a very slight erythema (score of 1) was evident on 1 ear of 1 animal on day 2, on both ears for all animals on day 3 and only 1 ear of 1 animal on day 6. Very slight oedema (score of 1) was present on 1 ear of 2 animals on day 3 and desquamation was present on both ears for all animals on day 6.

According to the criteria of the CLP Regulation, an SI index of > 3 is required for a substance to be regarded as a skin sensitiser. This was not the case at any concentration of bixlozone tested in this study: treatment with 5 %, 10 % and 25 % bixlozone resulted in stimulation index values of 1.13, 1.32 and 1.57, respectively. The positive control produced an SI value of 4.83 thus confirming the validity of the study. The negative control gave the expected result.

Table B 6.2.6.1: Individual and mean dpm values

Dose level	Animal #	dpm	dpm minus background[1]	Mean	Std. Dev.	SI[2]	SI > 3
Vehicle control	3601	620.54	540.53	494.09	58.45	-	-
	3602	620.73	540.72				
	3603	483.12	403.11				
	3604	596.02	516.01				
	3605	550.11	470.10				
Positive control (25 % HCA)	3606	2512.89	2432.88	2388.93	694.70	4.83	Yes
	3607	3036.25	2956.24				
	3608	3257.12	3177.11				
	3609	1825.08	1745.07				
	3610	1713.37	1633.36				
5 % test substance in AOO	3611	512.88	432.87	558.55	128.63	1.13	No
	3612	794.56	714.55				
	3613	755.00	674.99				
	3614	594.52	514.51				
	3615	535.85	455.84				
10 % test substance in AOO	3616	835.38	755.37	652.99	73.11	1.32	No
	3617	772.17	692.16				
	3618	695.74	615.73				
	3619	716.47	636.46				
	3620	645.23	565.22				
25 % test substance in AOO	3621	986.17	906.16	776.61	185.97	1.57	No
	3622	1116.36	1036.35				
	3623	727.78	647.77				
	3624	684.07	604.06				
	3625	768.73	688.72				

HCA: alpha-hexyl-cinnamaldehyde; AOO: acetone/olive oil (4:1 v/v)

[1] Values analysed for outliers, Grubbs 1969. Background = 80.01

[2] Stimulation Index = Average dpm of test substance/average dpm of vehicle

Conclusion

Bixlozone was not found to be a skin sensitiser under the conditions of the mouse local lymph node assay (LLNA) up to the maximum attainable concentration and hence no classification is required according to Regulation (EC) N°1272/2008.

Table B 6.2.6.2: Summary of the mouse local lymph node assay (LLNA)

Method, Species, Guideline	Test substance, doses	Results
Mouse local lymph node assay (LLNA) Mice, CBA/J, females, 2/group (preliminary irritation), 5/group (main test), 5/group (vehicle and positive control) Guideline: OECD 429 (2010) Deviations: none GLP [redacted] (2014f) **Acceptable**	F9600 technical, batch PL13-0203 Purity: 98.5 % Vehicle: acetone/olive oil Doses: 5 %, 10 % & 25 %	No dermal irritation observed for any of the vehicle control group sites or any of the test group sites. SI: 1.13, 1.32 & 1.57 at 5 %, 10 % & 25 %,respectively Positive & negative controls gave the expected results. No classification

B.6.2.7. Phototoxicity

Regulation (EC) N°283/2013 states that an *in vitro* phototoxicity study shall be required where the active substance absorbs electromagnetic radiation in the range 290 - 700 nm and is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution. If the ultraviolet/visible (UV/VIS) molar extinction/absorption coefficient of the active substance is less than 10 L × mol $^{-1}$ × cm $^{-1}$, no toxicity testing is required.

The UV/VIS absorption spectrum of bixlozone technical (Batch PL14-0163, purity 99.8 %), indicating the wavelengths at which bixlozone may be susceptible to photochemical degradation, was determined in a GLP and OECD compliant Guideline 101 study (Report N° WP36VB; Cowlyn, N. (2017)) as 200-800 nm. The data showed that under neutral, acidic and basic conditions, absorption was minimal at 290 nm (λ max under neutral condition at 999.2 mg/L: 290 nm (ε = 4.64 L mol^{-1} cm^{-1}) and that absorption did not occur in the range beyond 290 up to 700 nm. Please refer to Section B.2.4 (B.2.4. SPECTRA (UV/VIS, IR, NMR, MS), MOLAR EXTINCTION AT RELEVANT WAVELENGTHS, OPTICAL PURITY) for more details.

Since bixlozone shows no significant absorption of electromagnetic radiation above 290 nm and since the ultraviolet/visible molar extinction/absorption coefficient of the substance is less than 10 L × mol $^{-1}$ × cm $^{-1}$, no phototoxicity testing is required.

B.6.2.8. Summary of acute toxicity

The acute toxicity of bixlozone was investigated *in vivo* via the oral, dermal and inhalation routes. The skin irritating potential of bixlozone was investigated in the *in vitro* skin irritation test (SIT) using the Epiderm™ skin model (OECD Guideline 439) and in the *in vivo* study in rabbits (OECD Guideline 404). Two studies were conducted to investigate the eye irritating potential of bixlozone: the *in vitro* Epiocular™ eye irritation test (OECD Guideline 492), aiming to identify test items not classified for eye irritation / damage, and the *in vivo* study in rabbits. The skin sensitisation potential of bixlozone was evaluated in the LLNA up to the maximum attainable concentration of 25 % w/w. Bixlozone showed no significant absorption of electromagnetic radiation above 290 nm and the ultraviolet/visible molar extinction/absorption coefficient of the substance was less than 10 L × mol $^{-1}$ × cm $^{-1}$; thus no *in vitro* phototoxicity testing is required.

All of the studies mentioned above were conducted according to standard OECD Test Guidelines and were GLP compliant. Bixlozone was shown to be of low oral, dermal and inhalation acute toxicity and thus no

classification according to Regulation GB/NI Nº 1272/2008 is required for these endpoints. It was also demonstrated that bixlozone was not a skin or eye irritant and not a skin sensitiser according to CLP Criteria. A phototoxicity test is not required.

The table below provides an overview of the available acute toxicity studies.

Table B 6.2.8.1: Summary of bixlozone acute toxicity data, with classification according to Regulation GB/NI No 1272/2008

Study and acceptability	Result	Reference	Classification according to Reg. GB/NI No 1272/2008
Acute oral, rat ***Acceptable***	Oral LD_{50} > 2000 mg/kg bw	[redacted] (2014a)	No classification
Acute dermal, rat ***Acceptable***	Dermal LD_{50} > 2000 mg/kg bw	[redacted] (2014b)	No classification
Acute inhalation, rat ***Acceptable***	Inhalation 4hr LC_{50} > 2.11 mg/L (maximum attainable concentration)	[redacted] (2014c)	Acute Tox 4 (H332)
Skin irritation, *in vitro* (Epiderm™ skin model) ***Acceptable***	Not a skin irritant	Costin, GE. (2018)	No classification
Skin irritation, rabbit ***Unnecessary and not relied upon***	Slightly irritating; cleared within 72 hrs; insufficient for classification	[redacted] (2014d)	No classification
Eye irritation *in vitro* (Epiocular™ eye model) ***Supplementary. Potential false positive with a requirement for further testing'***	Eye irritant	Wilt, N. (2018)	Not applicable
Eye irritation, rabbit ***Acceptable***	Minimal eye irritation observed, cleared within 24 hrs	[redacted] (2014e)	No classification
Skin sensitization, mice (LLNA) ***Acceptable***	Negative up to 25% w/w (maximum attainable concentration)	[redacted] (2014f)	No classification
Phototoxicity test ***Not required***	Not applicable	Not applicable	Not applicable

B.6.3. SHORT-TERM TOXICITY

The short-term oral toxicity of bixlozone has been investigated in rats, mice and dogs in GLP and guideline studies. Rats and mice were tested via the oral dietary route after 28 and 90 days' exposure. In dogs, the animals were tested for 28 days via the oral dietary route and for 90 days and 12 months using oral capsules. In addition to these studies, in this Section, 7-day non-GLP dietary palatability studies conducted in each species and a 7-day study using capsules in dogs are presented. Since these studies were well-conducted, they are considered sufficiently reliable to contribute to the overall picture of the repeated-dose toxicity of bixlozone and are thus presented in this Section of the DAR.

Considering other routes of exposure a short-term 21-day dermal toxicity study in rats was conducted.

The main target organ of toxicity identified in all species was the liver. The toxicological significance of the effects observed on the liver has been assessed by HSE using a weight of evidence (WoE) approach, with the aim to make a clear distinction between adverse effects to those which are potentially adaptive. This has been carried out in line with the TAB (Technical Agreements for Biocides) entry[4], agreed at the Biocide WG-IV-2018 meeting (WGIV2018_TOX_6-2). HSE considers the approach described in this document is adequate to characterise the toxicity profile of bixlozone since this paper is based on several international reviews of liver effects (JMPR 2006 and 2015) and describes a detailed WoE approach for the evaluation of liver effects in repeated-dose toxicity studies. In short it depicts how hepatocellular hypertrophy is typically related to increased functional capacity of the liver which allows the maintenance of homeostasis in the organism after xenobiotic exposure. A general increase in the size of the liver is observed, owing to cell enlargement and fluid accumulation; this is considered a potentially beneficial, adaptive response. However, there is the potential that the capacity of the homeostatic mechanisms may be exceeded and in these cases the organism would be unable to return to its previous state once exposure has ended (thus constituting an adverse response). Hypertrophy as an adaptive response should not be accompanied by adverse histopathology (necrosis, apoptosis, pigment deposition or hyperplasia), or by substantial changes in clinical chemistry indicative of liver toxicity (decreased albumin or increased activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), bilirubin or cholesterol). In line with the TAB entry, relative liver weight increases up to 15 %, which are not accompanied by other signs of liver dysfunction, will be considered by the RMS to be an adaptive rather than an adverse response. However, a change in relative liver weight of greater than 15 % (in the absence of other adverse findings) will be regarded as the threshold at which effects on the liver have exceeded an adaptive response and have developed into adversity.

B.6.3.1. Oral 7-day studies

Three non-GLP supplementary 7-day oral dietary studies, investigating the palatability of the test substance in each species were conducted. Since these studies were well-conducted, they are considered to be sufficiently reliable to contribute to the overall picture of the repeated-dose toxicity of bixlozone and thus are presented below.

B.6.3.1.1. *Oral dietary 7-day palatability study in rats*

Study	A 7-Day Oral (Dietary) Palatability Study of F9600 Technical in Sprague Dawley Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted], (2015d)
Date performed	Jan 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105106
Guideline(s)	None This study was conducted according to testing facility SOPs and the study protocol as approved by the Sponsor.
Deviations from the guideline	N/A
GLP	None; however this study was conducted according to testing facility SOPs and the study protocol as approved by the Sponsor.

[4] https://webgate.ec.europa.eu/s-circabc/d/a/workspace/SpacesStore/83e2fb72-5d1f-4ada-be15-2246109e65d4/Interpretation%20of%20liver%20effects.pdf

Test material	F9600 technical, batch PL13-0385 Purity: 99.2%
Method of analysis	Validation is not required since this is not a GLP study to rely on.
Study acceptable	Yes but as supplementary information only

Material and Methods

Bixlozone technical was administered *ad libitum* in the diet to Crl:CD(SD) rats (5/sex/group) for seven consecutive days at 4000, 7000, and 12000 ppm with a concurrent control group. Based on food consumption these dietary concentrations corresponded to dose levels of 441, 698, and 1067 mg/kg bw/day for males and 434, 763, and 1250 mg/kg bw/day for females. The selected dosage levels were not expected to cause lethality in the study animals based on information from a closely related analogue compound. All animals were observed twice daily for mortality and morbidity and clinical examinations once daily. Following euthanasia, the liver from each animal was collected, weighed and examined for macroscopic findings.

Results

There were no reported deaths; the only clinical sign of toxicity noted was the presence of a yellow material around the urogenital area of males from 7000 ppm. This is not considered adverse by HSE. Statistically significant lower mean food consumption values were noted for the 7000 (-16 %) and 12000 ppm (-28 %) group males from study days 0-7 when compared to the control group. This finding correlated with dose-dependent decreases in body weight gain reaching statistical significance at 7000 ppm and 12000 ppm (-24 and -45.5 % respectively) in males and at 12000 ppm (-45.5 %) in females in comparison to controls.

Statistically significant higher mean absolute and relative liver weights were noted in both sexes of all treated groups, reaching 30 % (absolute) and 47 % (relative) in males and 37 % (absolute) and 45 % (relative) in females at top dose (12000 ppm). The increases in relative liver weight were > 15 % compared to controls for all treated groups in both sexes and showed a clear dose-related increase. There were no test substance-related macroscopic findings at the scheduled necropsy.

Table B 6.3.1.1: Summary of key findings in the 7-day palatability study in rats

	Males				Females			
Dose (ppm)	0	4000	7000	12000	0	4000	7000	12000
Dose (mg/kg bw/day)	0	441	698	1067	0	434	763	1250
Body weight								
Final body weight (g)	251 ± 13.8	249 ± 14.4	238 ± 19.1	**223 ± 14.4***	167 ± 9.0	173 ± 18.6	162 ± 9.7	158 ± 10.8
% difference from control		-1	-5	**-11**		+4	-3	-5.4
Body weight gain 0-7 days (g)	66 ± 3.3	60 ± 3.2	**50 ± 6.6****	**36 ± 6.7****	22 ± 6.2	25 ± 5.2	17 ± 3.5	**12 ± 6.0****
% difference from control		-9	**-24**	**-45**		+14	-23	**-45**
Food consumption								
0 – 7 days (g/animal/day)	25 ± 1.5	24 ± 1.9	**21 ± 1.1****	**18 ± 0.8****	17 ± 1.1	17 ± 3.5	17 ± 1.6	16 ± 4.4
% difference from control		-4	**-16**	**-28**		0	0	-6
Liver weight								
Absolute liver weight (x)	13.3 ± 1.57	**17.9 ± 1.3****	**17.3 ± 1.7****	**17.3 ± 1.86****	8.60 ± 0.74	**11.39 ± 1.02****	**11.25 ± 0.99****	**11.80 ±1.77****
% difference from control		**+34.5**	**+30**	**+30**		**+32**	**+31**	**+37**
Relative liver weight to body weight	5.29 ± 0.38	**7.17 ± 0.30****	**7.24 ± 0.23****	**7.75 ± 0.59****	5.15 ± 0.296	**6.61 ± 0.17****	**6.93 ± 0.22****	**7.46 ± 0.796****
% difference from control		**+36**	**+37**	**+47**		**+28.5**	**+35**	**+45**

* = statistically-significant from the control group at 0.05 using Dunnett's test
** = statistically-significant from the control group at 0.01 using Dunnett's test

Conclusion

In a non-GLP and non-OECD compliant palatability study, bixlozone technical was administered in the diet to Crl:CD(SD) rats for seven consecutive days. In males the adverse effects observed consisted of a greater than 10 % lower body weight gain and correlating lower food consumption at doses ≥ 7000 ppm. In females the 45.5

% lower body weight gain observed at 12000 ppm was unrelated to effects on food consumption, indicating that bixlozone technical exerted a direct toxic effect on body weight at least in female rats. In males, although a correlation between food consumption and decreases in body weight gain was observed, without further investigations (e.g. studies with other methods of administration), it cannot be excluded that the effects on body weight were primary toxic effects of the substance rather than the consequence of palatability issues. Adverse changes in relative liver weight (> 15 %) compared to controls were observed for all treated groups in both sexes and showed a dose-related increase.

[redacted], (2015d)

B.6.3.1.2. *Oral dietary 7-day palatability study in mice*

Study	A 7-Day Oral (Dietary) Palatability Study of F9600 in CD-1 Mice
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2015c)
Date performed	Jan 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105107
Guideline(s)	None; however, this study was conducted according to testing facility SOPs and the study protocol as approved by the Sponsor.
Deviations from the guideline	N/A
GLP	No
Test material	F9600 technical, batch PL13-0385 Purity: 99.2%
Method of analysis	Validation is not required since this is not a GLP study to rely on.
Study acceptable	Yes but as supplementary information only

Material and Methods

Male and female Crl:CD-1(ICR) mice (5/sex/group) were exposed to bixlozone technical *ad libitum* in the diet for seven consecutive days. Dietary concentrations of 0, 2000, 4000 and 6000 ppm were used. These corresponded to dosage levels of 404, 960, and 1348 mg/kg bw/day for males, and 476, 886, and 1460 mg/kg bw/day for females, based on food consumption. The doses selected were not expected to cause lethality in the study animals based on information from a closely related analogue compound. All animals were observed twice daily for mortality and morbidity and clinical examinations once daily. Following euthanasia the liver from each animal was collected, weighed and examined for macroscopic findings.

Results

There were no treatment-related deaths or clinical signs of toxicity. There were no effects on food consumption or palatability noted in any test substance-treated group compared to the control group in both sexes. A dose-response reduction in the mean body weight was seen in males, reaching statistical significance (-11 % compared to controls) at the top dose of 6000 ppm, whilst a dose-response reduction in the mean body weight gain was seen in females up to the top dose, reaching -42 % compared to controls at 6000 ppm.

Relative liver weights were increased in both sexes in a dose-response manner compared to controls, reaching statistical significance in males at 6000 ppm (+16.5 %) whilst in females increases (but not reaching statistical significance) of 13.5, 20 and 24 % were observed at 2000, 4000 and 6000 ppm respectively. There were no macroscopic findings in the liver.

Table B 6.3.1.2: Summary of key findings in the 7-day palatability study in mice

	Males (n = 5)				Females (n = 5)			
Dose (ppm)	0	2000	4000	6000	0	2000	4000	6000
Dose (mg/kg bw/day)	0	404	960	1348	0	476	886	1460
Body weight								
Final body weight (g)	251 ± 13.8	249 ± 14.4	238 ± 19.1	**223 ± 14.4***	167 ± 9.0	173 ± 18.6	162 ± 9.7	158 ± 10.8
% difference from control		-1	-5	**-11**		+4	-3	-5.4
Body weight gain 0-7 days (g)	2.4 ± 0.46	2.1 ± 0.46	3.3 ± 0.89	2.3 ± 0.74	1.7 ± 0.64	1.9 ± 0.64	1.2 ± 0.72	0.9 ± 0.76
% difference from control		-12.5	-+37.5	-4		+12	-29	-47
Food consumption								
0 – 7 days (g/animal/day)	5.8 ± 0.76	6.4 ± 0.64	7.2 ± 1.01	6.5 ± 0.75	5.8 ± 0.91	5.6 ± 0.52	5.5 ± 0.72	6.1 ± 1.90
% difference from control		+10	+24	+12		-3	-5	+5
Liver weight								
Absolute liver weight (x)	13.3 ± 1.57	**17.9 ± 1.3****	**17.3 ± 1.7****	**17.3 ± 1.86****	5.8 ± 0.74	**11.39 ± 1.02****	**11.25 ± 0.99****	**11.80 ±1.77****
% difference from control		**+34.5**	**+30**	**+30**		**+32**	**+31**	**+37**
Relative liver weight to body weight	5.29 ± 0.38	**7.17 ± 0.30****	**7.24 ± 0.23****	**7.75 ± 0.59****	5.15 ± 0.296	**6.61 ± 0.17****	**6.93 ± 0.22****	**7.46 ± 0.796****
% difference from control		**+36**	**+37**	**+47**		**+28.5**	**+35**	**+45**

* = statistically-significant from the control group at 0.05 using Dunnett's test
** = statistically-significant from the control group at 0.01 using Dunnett's test

Conclusion

In a palatability study, bixlozone was fed to Crl:CD-1(ICR) mice for 7 consecutive days. In males a dose-related reduction in mean body weight was observed, reaching statistical significance (-11 % compared to controls) at top-dose (6000 ppm). A dose-related reduction in mean body weight gain reaching -42 % (compared to controls) at top-dose was noted in females. A test substance-related increase in relative liver weights was observed in both sexes in a dose-related manner, exceeding 15 % at top dose in males and from 4000 ppm in females.

This study shows that body weight effects in mice are not secondary to reductions in food consumption and therefore are not due to palatability issues.

[redacted] (2015c)

B.6.3.1.3. *Oral dietary 7-day palatability study in dogs*

Study	A 7-Day Oral (Dietary) Palatability Study of F9600 Technical in Beagle Dogs
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2015e)
Date performed	Mar 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105126
Guideline(s)	None This study was conducted according to testing facility SOPs and the study protocol as approved by the Sponsor.
Deviations from the guideline	N/A
GLP	None; however, this study was conducted according to testing facility SOPs and the study protocol as approved by the Sponsor.
Test material	F9600 technical, batch PL13-0385 Purity: 99.2%
Method of analysis	Validation is not required since this is not a GLP study to rely on.
Study acceptable	Yes but as supplementary information only

Material and Methods

Bixlozone technical was administered *ad libitum* in the diet of Beagle dogs (2/sex/group) for seven consecutive days, initially at concentrations of 0, 2500, 5000 and 10000 ppm. Since no clinical findings were observed at the top dose, an additional group (2/sex) was administered a higher dose of 30000 ppm for seven consecutive days. These dietary concentrations corresponded with dosage levels of 67, 185, 292, and 818 mg/kg bw/day for males, and 79, 187, 244, and 716 mg/kg bw/day for females.

Animals were not sacrificed on completion of the study thus the clinical signs, body weight, food consumption, haematology and clinical (serum) chemistry could be measured.

Results

All animals survived to the end of the study. The presence of a clear material around the mouths of males at 10000 ppm and of females from 5000 ppm was the only notable clinical sign noted.

Lower food consumption and body weights were observed at 30000 ppm for the first 2-3 days in both sexes. Subsequently, improvement in food consumption led to an overall body weight gain that was comparable with controls; nevertheless, the mean body weight of these dogs was still lower than controls by the end of the study. Mean body weights and body weight gains were unaffected in animals treated with bixlozone technical at lower doses.

Table B 6.3.1.3: Summary of key findings in the 7-day palatability study in dogs

	Males (n = 2)					Females (n = 2)				
Dose (ppm)	0	2500	5000	10000	30000	0	750	2500	5000	30000
Dose (mg/kg bw/day)	0	67	185	292	818	0	79	187	244	716
Body weight										
Body weight day 3	9.5	10.4	8.4	10.6	-	8.4	9.0	8.3	8.6	-
Body weight day 3	11.0	-	-	-	9.8	8.2	-	-	-	7.7
% difference from control		+9.5	-12	+12	-11		+7.1	-1.2	+2.4	-6
Final body weight (kg)	9.5	10.1	8.5	10.7	-	8.4	8.9	8.3	8.7	-
Final body weight (kg)	10.8	-	-	-	10.0	8.0	-	-	-	7.7
% difference from control		+6.3	-10.5	+13	-7.4		+6	-1.2	+3.6	-3.8
Body weight gain 0-8 days (g)	-0.1	0.0	0.1	-0.1	-0.1	0.1	0.2	-0.2	0.2	-0.2
Food consumption										
Day 0 to 1 (g/animal/day)	247	267	327	204		276	301	243	214	
% difference from control	-	+8	+32	-17		-	+9	-11	-22	
Day 0 to 1 (g/animal/day)	360				113	382				7
% difference from control	-	-	-	-	-69	-	-	-	-	-98
Day 6 to 7 (g/animal/day)	389	320	312	304		270	267	346	150	
% difference from control		-17	-20	-21			-1	+28	-44	
Day 6 to 7 (g/animal/day)	348				278	370	-	-	-	218
% difference from control	-	-	-	-	-20	-	-	-	-	-41

* = statistically-significant from the control group at 0.05 using Dunnett's test
** = statistically-significant from the control group at 0.01 using Dunnett's test

There was no notable treatment-related effect on haematological and serum chemistry parameters, including for parameters associated with liver function such as AP, ALT and AST, in any treated group in both sexes.

Conclusion

In a palatability study, dietary administration of bixlozone technical to Beagle dogs for 7 consecutive days was well tolerated up to 10000 ppm and transient reductions in food consumption and body weight loss were noted during the first three study days in both sexes at the top dose of 30000 ppm. There was no notable treatment-related effect on haematological and serum chemistry parameters in any treated group in both sexes. It is possible the body weight effects seen at 30000 ppm (716 and 818 mg/kg bw/day in M/F) were due to palatability issues since substantial decrease in food consumption was observed at top-dose during the length of the study.

[redacted] (2015e)

B.6.3.1.4. *Oral capsule 7-day study in dogs*

Study	A 7-Day Oral (Capsule) Toxicity Study of F9600 Technical in Beagle Dogs.
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016b)
Date performed	Dec 2014
Test facility	[redacted] & [redacted]
Report reference	Study no. [redacted]-105154
Guideline(s)	None This study was conducted according to testing facility SOPs and the study protocol as approved by the Sponsor.
Deviations from the guideline	N/A
GLP	None; however this study was conducted according to testing facility SOPs and the study protocol as approved by the Sponsor.
Test material	F9600 technical, batch PL14-0049 Purity: 96.0%
Method of analysis	Validation is not required for this non-GLP study owing to route of administration used (capsules).
Study acceptable	Yes but as supplementary information only

Material and Methods

To investigate whether the initial reductions in food consumption and body weights observed in dogs administered bixlozone orally in the diet for 7 days ([redacted] 2015e) and 28 days ([redacted], 2016a – see later), a 7-day oral study using capsules was conducted.

Bixlozone technical was administered once daily in capsules to 4 groups of Beagle dogs (2/sex/group) for seven consecutive days at doses of 0 (vehicle: capsules without test item), 150, 350 and 550 mg/kg bw/day. Dose levels were selected based on results from the dietary studies conducted in dogs.

Animals were not sacrificed on completion of the study thus clinical signs, body weight, food consumption, haematology and clinical (serum) chemistry could be measured.

Results

All animals survived to the end of the study and did not display any notable clinical sign up to the top dose of 550 mg/kg bw/day compared to the control groups. Mean body weights, body weight gains and food consumption were unaffected by treatment during the study.

There was no notable treatment-related effect on haematological and serum chemistry parameters in any treated group in both sexes; no signs of test substance-related effects on liver function were noted since levels of AP, ALT and AST were found similar between the test substance-treated groups and the control groups.

Conclusion

Oral (capsule) administration of bixlozone technical in male and female Beagle dogs for 7 consecutive days was well tolerated at doses up to 550 mg/kg bw/day. No treatment-related findings were observed during the study

period up to the highest dose tested. This study confirmed that in dogs, palatability issues occurred initially at relatively high dietary doses.

[redacted] (2016b)

B.6.3.2. Oral 28-day studies

Three GLP oral (dietary) studies in which bixlozone was administered to rats, mice and dogs for 28 days are available.

B.6.3.2.1. *Oral 28-day study in rats*

The potential toxicity of bixlozone after 28 days' oral administration has been investigated in rats. Toxicokinetics evaluations were included in the study; they are reported in more details in the ADME Section of the DAR (Section B.6.1.1.3).

Study	A 28-Day Oral (Dietary) Toxicity and Toxicokinetic Study of F9600 Technical in Sprague Dawley Rats.
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2015a)
Date performed	Jan-Feb 2014
Test facility	[redacted] & [redacted]
Report reference	Study no. [redacted]-105108
Guideline(s)	OECD Guideline 407 (2008)
Deviations from the guideline	None
GLP	Yes
Test material	F9600 technical; Batch PL13-0385 Purity 99.2%
Method of analysis	[redacted], *2014;* [redacted]*; Study No.* [redacted]*-105110) and the data are presented in Volume 3, Section CA B.4, Point CA 4.1.2(c).* *The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to the lack of explanation of the use of a non-linear calibration.* However, the method is considered sufficient for regulatory purposes.
Study acceptable	Yes

Material and Methods

In a GLP and guideline compliant study, bixlozone technical (purity 99.2%; batch PL13-0385) was administered via the diet for 28 consecutive days to Sprague Dawley rats (5/sex/group) at doses of 750, 2500, 5000 and 10000 ppm. These doses equated to intakes of 57, 182, 359 & 740 mg/kg bw/day in males and 61, 193, 379 & 733 mg/kg bw/day in females. Concurrent control groups received basal diet only.

Results

Survival and clinical observations

All animals survived to the end of the study and no treatment-related clinical signs of toxicity were recorded.

Mean body weight and body weight gain (Table B 6.3.2.1)

Bixlozone had a notable effect on mean body weights and overall body weight gain, predominantly in females from 2500 ppm and in males at the top dose. There was a dose-dependent decrease in mean body weight and body weight gain of females by the end of the study from 2500 ppm, though statistical significance was only reached in the 10000 ppm group (-59 % lower than controls for body weight gain and -18 % for body weight). In males, an initial decrease in body weight gain of the high-dose group (-44 % on days 0-7) was not maintained throughout the study; cumulative body-weight gain of top dose males by the end of the study was still -14 % lower than the corresponding control (albeit without statistical significance).

Overall, there was an adverse effect of treatment on body weight and body weight gain in females from 2500 ppm and in males at the top dose of 10000 ppm.

Table B 6.3.2.1: Summary of mean body weights and body-weight-gain in the 28-day rat study

	Males					Females				
Dose (ppm)	**0**	**750**	**2500**	**5000**	**10000**	**0**	**750**	**2500**	**5000**	**10000**
(mg/kg bw/d)	**0**	**57**	**182**	**359**	**740**	**0**	**61**	**193**	**379**	**733**
Mean Body weights										
Day 7	315 ± 24.9	308 ± 20.6	294 ± 18.6	297 ± 31.0	289 ± 17.3	198 ± 6.4	197 ± 14.1	191 ± 5.0	184 ± 12.6	170** ± 13.1
Day 14	369 ± 27.7	355 ± 26.9	335 ± 24.8	347 ± 37.5	340 ± 16.7	220 ± 6.9	216 ± 15.9	207 ± 7.3	198* ± 15.6	183** ± 10.4
Day 14 (% difference from control)	-	-3.8	-3.8	-6.0	-7.9	-	-1.8	-5.9	-10	**-17****
Day 21	404 ± 27.5	396 ± 29.9	372 ± 28.9	386 ± 38.6	382 ± 18.6	235 ± 8.2	232 ± 16.7	220 ± 6.2	213* ± 16.6	191** ± 14.3
Day 27	438 ± 35.0	424 ± 23.6	403 ± 33.5	418 ± 40.1	411 ± 15.9	242 ± 9.6	238 ± 17.3	226 ± 7.2	223 ± 16.6	198** ± 12.6
Day 27 (% difference from control)	-	-3.2	-8.0	-4.6	-6.2	-	-1.65	-6.6	-7.85	**-18****
Cumulative body weight gain										
Days 0-7	54 ± 6.7	51 ± 9.3	43 ± 8.9	41 ± 6.2	30** ± 10.9	26 ± 5.0	21 ± 6.1	20 ± 5.4	12** ± 3.0	1** ± 5.3
Days 7-14	55 ± 4.6	47 ± 7.6	41* ± 7.1	49 ± 8.3	51 ± 2.9	22 ± 2.6	19 ± 5.8	16 ± 3.5	14 ± 4.1	13* ± 5.3
Days 14-21	34 ± 6.0	41 ± 4.9	37 ± 5.4	40 ± 3.1	42 ± 6.4	16 ± 2.7	16 ± 4.8	13 ± 3.3	15 ± 1.1	9.0* ± 5.0
Days 0-14	109 ± 10.8	98 ± 16.5	84* ± 15.2	90 ± 13.3	81* ± 12.2	47 ± 7.3	40 ± 10.3	36 ± 4.7	27** ± 5.5	14** ± 5.8
Days 0-21	143 ± 10.6	139 ± 18.2	121 ± 19.7	130 ± 14.9	123 ± 15.9	63 ± 9.8	56 ± 13.6	49 ± 6.1	41** ± 6.5	23** ± 8.6
Days 0-27 (g)	177 ± 16.5	167 ± 14	152 ± 24	161 ± 18	153 ± 16	70 ± 11	63 ± 16	54 ± 11	52 ± 8	**29** ± 10**
Days 0-27 (% difference from control)	-	-5.6	-14	-9.0	-14	-	-10	-23	-26	**-59****

* = statistically-significant from the control group at 0.05 using Dunnett's test
** = statistically-significant from the control group at 0.01 using Dunnett's test

Food consumption (Table B 6.3.2.2)

Statistically significant lower food consumption was noted in females at 2500 ppm on days 0-14 (up to 12 %), at 5000 ppm on days 0-21 (up to 24 %) and at 10000 ppm for the duration of the study to an extent ranging from -41 % on days 0-7 to -22 % on days 14-27; this would account in part for the significantly lower overall body weight gain observed in this dose-group. Food consumption in males was only statistically significantly lower than controls on days 0-7 (-16 % lower at 5000 ppm and -20 % lower at 10000 ppm) and was comparable with controls throughout the remainder of the study period.

Overall, there were adverse, treatment-related effects on food consumption from 2500 ppm in females and from 5000 ppm in males.

Table B 6.3.2.2: Food consumption in the 28-day rat study

Food consumption (g/animal/day)										
	Males					Females				
Dosage (ppm)	**0**	**750**	**2500**	**5000**	**10000**	**0**	**750**	**2500**	**5000**	**10000**
(mg/kg bw/d)	**0**	**57**	**182**	**359**	**740**	**0**	**61**	**193**	**379**	**733**
Days 0-7	25 ± 2.0	24 ± 1.9	22 ± 1.7	**21* ± 1.8**	**20** ± 3.0**	17 ± 0.4	16 ± 0.8	**15** ± 0.4**	**13** ± 0.7**	**10** ± 1.5**
% from control	-	-4	-12	**-16**	**-20**	-	-6	**-12**	**-23.5**	**-41**
Days 7-14	27 ± 1.8	26 ± 1.6	24 ± 2.3	25 ± 2.2	26 ± 1.1	18 ± 0.5	18 ± 1.3	**16* ± 0.8**	**15** ± 1.1**	**15** ± 0.8**
% from control	-	-4	-11	-7.4	-4	-	0	**-11**	**-17**	**-17**
Days 14-21	26 ± 1.8	27 ± 2.1	24 ± 2.2	25 ± 2.0	26 ± 2.3	18 ± 0.5	17 ± 1.5	16 ± 1.1	**15* ± 1.1**	**14** ± 0.8**
% from control	-	+4	-8	-4	0	-	-5.5	-11	**-17**	**-22**
Days 21-27	28 ± 2.4	28 ± 2.5	26 ± 2.5	26 ± 2.2	26 ± 2.2	18 ± 1.3	18 ± 1.6	16 ± 1.1	16 ± 0.8	**14** ± 1.2**
% from control	-	0	-7	-7	-7	-	0	-11	-11	**-22**

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test

Haematology, clinical chemistry and urinalysis parameters (Table B 6.3.2.3)

No biologically relevant changes were noted in urinalysis or haematology parameters.

With regard to clinical chemistry, metabolic parameters showed a dose-dependent and statistical significantly higher serum cholesterol levels at 10000 ppm in males (+79 %) and females (+91 %) and also at 5000 ppm in females (+43 %), indicating a possible impairment of liver function. Cholesterol was also increased (but not statistically significantly) at 2500 ppm in females (by +15 %) and at 5000 ppm in males (by +31 %). However, due to the magnitude of the change at these dose levels, the increase is not considered adverse. Triglycerides were also increased from the mid-doses in both sexes, reaching statistical significance at top-dose in females only (+86 %).

With regard to serum proteins, statistically significant higher total protein levels were noted in males from 5000 ppm owing to statistically significant increase in globulin from that dose and albumin at the top-dose. In females, significant higher globulin was seen across all treatment groups, resulting in a consistently lower albumin/globulin (A/G) ratio and a higher (but not statistically significant) total protein levels. Due to a lack of dose-response, the relation to treatment of these effects in females remains unclear, with the possible exception of the findings at the two highest doses. It is also noted that a higher serum calcium level is observed at top-dose in males (+3.9 %) and females (+5.7 %).

Statistically significant lower alkaline phosphatase (ALP) values compared to controls were only observed in females at 750, 2500 and 5000 ppm, with a value comparable to controls at top-dose. However these changes are decreases without a dose-response pattern and they are not accompanied with changes in any of other relevant liver enzymes measured in this study. Overall, they are not considered to be a relevant indication of liver function disruption.

With regard to parameters indicators of renal function there was dose-dependent increase in blood urea nitrogen (BUN) reaching statistical significance at top-dose in females, although no parallel increase was seen for creatinine. No such changes were seen in the males. This finding may be an early indication of renal function disruption.

Overall, there were adverse changes in clinical chemistry parameters indicative of metabolic changes and possible liver damage from 5000 ppm in both sexes as well as an early indication of renal function disruption at top-dose in females.

Table B 6.3.2.3: Selected clinical chemistry findings in the 28-day rat study

	Males					Females				
Dose (ppm)	**0**	**750**	**2500**	**5000**	**10000**	**0**	**750**	**2500**	**5000**	**10000**
(mg/kg bw/d)	**0**	**57**	**182**	**359**	**740**	**0**	**61**	**193**	**379**	**733**
	Serum chemistry									
Cholesterol mg/dL	57±15.9	60±19.4	66±20.08	75±13.7	**102**±15.3**	70±21.7	69±18.4	80±6.2	**100*±10.8**	**134**±10.7**
Triglyceride (mg/dL)	46 ± 14.6	43 ± 14.3	43 ± 10.7	57 ± 21.4	70 ± 21.4	28 ± 5.5	31 ± 8.5	32 ± 4.3	33 ± 4.3	**52* ± 22.6**
Total protein (g/dL)	6.2 ± 0.33	6.5 ± 0.16	6.5 ± 0.29	**6.7* ± 0.23**	**7.0** ± 0.23**	6.8 ± 0.54	7.2 ± 0.21	7.2 ± 0.57	7.2 ± 0.17	7.4 ± 0.43
Albumin (g/dL)	3.7 ± 0.21	3.9 ± 0.09	3.9 ± 0.16	4.0 ± 0.22	**4.1**± 0.15**	4.2 ± 0.37	4.2 ± 0.08	4.3 ± 0.32	4.3 ± 0.11	4.4 ± 0.24
Globulin (g/dL)	2.5 ± 0.13	2.6 ± 0.13	2.6 ± 0.14	**2.7**± 0.05**	**2.8** ± 0.09**	2.6 ± 0.19	**3.0* ± 0.16**	**3.0* ± 0.26**	**3.0* ± 0.15**	**3.0* ± 0.22**
A/G Ratio	1.48 ± 0.045	1.48 ± 0.110	1.50 ± 0.000	1.42 ± 0.084	1.44 ± 0.055	1.64 ± 0.055	**1.42** ± 0.084**	**1.44** ± 0.055**	**1.42** ± 0.084**	**1.46** ± 0.089**
Calcium (mg/dL)	10.3 ± 0.24	10.3 ± 0.31	10.3 ± 0.24	10.4 ± 0.23	**10.7* ± 0.15**	10.6 ± 0.29	10.6 ± 0.11	10.7 ± 0.39	10.8 ± 0.33	**11.2* ± 0.23**
BUN (mg/dL)	14.0 ± 1.17	14.1 ± 0.85	13.5 ± 2.01	15.0 ± 2.97	13.8 ± 1.68	12.1 ± 1.83	13.2 ± 1.25	15.0 ± 0.63	15.7 ± 2.41	**17.6** ± 4.09**
ALP (U/L)	170 ± 28.2	156 ± 30.3	174 ± 14.6	159 ± 39.6	147 ± 19.4	130 ± 6.1	**99* ± 9.4**	**104* ± 13.5**	**81** ± 18.3**	125 ± 19.9

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test
g/dL grams/decilitre
mg/dL milligrams/decilitre
U/L international unit/litre
A/G ratio = Albumin / Globulin ratio; ALP = Alkaline Phosphatase; BUN = Blood Urea Nitrogen

Organ weight changes

With regard to organ weight changes, absolute liver weights were statistically significantly greater than controls in males at 10000 ppm (+56 %), and in females at 5000 ppm (+19 %) and 10000 ppm (+32 %). Relative liver weights were increased (statistically significant) in both sexes in comparison with controls at 2500, 5000 and 10000 ppm (+15.5, 23 & 65.5 % respectively in males and +17, 29 & 61 % respectively in females). The liver weight changes were also associated with histopathological signs (hepatocellular hypertrophy; further details below) indicative of an adaptive rather than an adverse effect from treatment; nevertheless HSE considers that the magnitude of the relative liver weight increases from 2500 ppm onwards (> 15 % from controls) constitutes an adverse effect in itself, regardless of the mechanism.

Relative kidney weights were increased by 14.5 % in males and 14 % in females at the top-dose, but there was no corresponding effect on the absolute kidney weights. HSE considers these changes treatment-related as a secondary consequence of the treatment-related effects on body weight, in particular considering that such effects are observed to a greater extent when the length of exposure is increased from 28 to 90 days ([redacted] (2016a)).

Absolute (but not relative) thymus weights were lower (by 27 %) than controls in females at 2500 and 10000 ppm but not at 5000 ppm; the lack of a dose-response suggests that this is unlikely to be related to treatment with bixlozone.

Overall, there were adverse treatment-related effects on relative liver weight from 2500 ppm and on kidney weight at the top dose in both sexes.

Table B.6.3.1.2.3. Selected organ weight changes from the 28-day rat study

	Males					Females				
Dosage (ppm)	**0**	**750**	**2500**	**5000**	**10000**	**0**	**750**	**2500**	**5000**	**10000**
(mg/kg bw/d)	**0**	**57**	**182**	**359**	**740**	**0**	**61**	**193**	**379**	**733**
	Selected organ weights									
Terminal body weight	408 ± 32.7	401 ± 26.8	377 ± 32.4	392 ± 40.7	386 ± 17.7	226 ± 9.4	223 ± 18.2	213 ± 9.0	209 ± 17.0	**185** ± 12.5**
% from control	-	-2	8	-4	-5	-	-1	-6	-7.5	**+18**
Liver (g)	12.90 ± 1.50	13.96 ± 1.12	13.76 ± 1.87	15.22 ± 1.28	**20.16** ± 1.47**	7.58 ± 0.39	8.22 ± 0.70	8.34 ± 0.73	**9.01** ± 0.65**	**10.02** ± 1.29**
% from control	-	+8	+7	**+18**	**+56**	-	+8	+10	**+19**	**+32**
R liver	-	+10	**+15.5****	**+23****	**+65.5****	-	+10	**+17****	**+29****	**+61****
Kidneys (g)	3.20 ± 0.42	3.13 ± 0.24	3.12 ± 0.37	3.20 ± 0.36	3.45 ± 0.16	1.89 ± 0.11	1.89 ± 0.14	1.78 ± 0.06	1.81 ± 0.15	1.77 ± 0.10
% from control	-	-2	-2.5	0	+8	-	0	-6	-4	-6
R kidneys	-	-0.1	+6	+4	**+14.5****	-	1.3	0.0	+4	**+14****
Thymus (g)	0.50 ± 0.05	0.48 ± 0.05	0.49 ± 0.13	0.50 ± 0.10	0.49 ± 0.10	0.48 ± 0.13	0.46 ± 0.03	**0.35** ± 0.04**	0.46 ± 0.06	**0.35** ± 0.06**
% from control	-	-4	-2	0	-2	-	-4	-27	-4	-27
R thymus	-	-4.0	+5	+1.6	+1.6	-	+2.4	-22	+4	-11

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test

(R) =% change in relative weight (organ weight to bodyweight) from control

Histopathology findings (Table B 6.3.2.4)

There were histopathological correlates found in the livers of male and female rats upon necropsy, at doses of 2500 ppm and above. Hepatocellular hypertrophy, characterised by an expansion of the hepatocellular cytoplasm with predominant centrilobular distribution, was generally defined as minimal or mild (one male of the high-dose group being defined as moderate). The findings showed a clear dose-related response both in incidence and severity. There was also some white discoloration of the liver noted upon macroscopic evaluation in 1/5 high-dose males and 1/5 females of the 5000ppm dose group. There were no other treatment-related macroscopic or histopathological findings, including those that would account for the sporadic changes in the weights of the thymus or the increases in kidney weights.

Overall, liver histopathological findings (hypertrophy) were observed in both sexes from 2500 ppm.

Table B 6.3.2.4: Selected microscopic findings in the 28-day rat study

	Males					Females				
Dosage (ppm)	**0**	**750**	**2500**	**5000**	**10000**	**0**	**750**	**2500**	**5000**	**10000**
(mg/kg bw/d)	**0**	**57**	**182**	**359**	**740**	**0**	**61**	**193**	**379**	**733**
	Microscopic findings									
Liver (N)	5	5	5	5	5	5	5	5	5	5
Hypertrophy, Hepatocellular	0	0	**3**	**5**	**5**	0	0	**4**	**5**	**5**
Minimal	-	-	**3**	**3**	-	-	-	**4**	**1**	-
Mild	-	-	-	**2**	**4**	-	-	-	**4**	**5**
Moderate	-	-	-	-	**1**	-	-	-	-	-

N =number of animals examined

Toxicokinetics ***(Error! Reference source not found.)***

Additional toxicokinetic investigations were included in the study; they are reported in detail in the ADME Section of the DAR (Section B.6.1.1.3). In short, they showed that the test substance exposure increased with the dose except for females on day 1 from 5000 ppm where exposure plateaued, indicating possible saturation of absorption. Females' exposure was higher than for the males especially at lower doses and for all rats, the exposure appeared to be lower on day 27 than on day 1 despite similar food consumption observed over the time of study. This possibly indicates an enhanced metabolism of the test substance in both sexes over time, with effects more pronounced in males compared to female rats. There was no indication that bixlozone accumulated upon repeated dosing.

Conclusion

In conclusion, in a GLP and guideline compliant study, dietary administration of bixlozone to Sprague Dawley rats for 28 days resulted in marked systemic toxicity at the top-dose of 10000 ppm in females, characterised by a reduction in cumulative body-weight gain of -59 % and a corresponding decrease in food consumption. Adverse effects on body weight, body weight gain and food consumption were also seen in females from 2500 ppm. Body weights and body weight gains were also affected (albeit less dramatically) in top dose males. Adverse increases in relative liver weights (> 15%) were seen in both sexes from 2500 ppm. These changes were associated with hepatocellular hypertrophy findings showing a clear dose-related response both in incidence and severity. In addition, changes in clinical-chemistry parameters indicative of metabolic changes and liver damage were seen from 5000 ppm in both sexes.

Relative kidney weight was also statistically significantly increased at the top dose in both sexes, which was accompanied with increased BUN levels reaching statistical significance in females at top-dose.

The toxicokinetics parameters showed that females' blood contained higher concentrations of bixlozone compared to males throughout the study and doses.. It may thus explain why more severe adverse effects were observed for females in this study compared to males.

Overall, a NOAEL of 750 ppm (equivalent to 57 and 61 mg/kg bw/d in males and females respectively) has been set by HSE from this study, based on relative liver weight increases at 2500 ppm (182/193 mg/kg bw/day in M/F) in both sexes (15.5 % and 17 % in males and females respectively) and adverse effects on body weight, body weight gain and food consumption seen in females his dose. A NOAEL of 5000 ppm has been proposed by the applicant, based on decreased food consumption and increased liver weights at 10000 ppm.

[redacted] (2015a)

Table B 6.3.2.5: Summary of the 28-day dietary study in the rat

Method, Species, test substance	Doses	NOAEL	Main adverse effects
28 day, dietary Rat, Crl :CD9(SD), males & females, 5/sex/toxicology group (Inc. control), 9/sex/toxicokinetic group (3/sex/control group) GLP OECD 407 (2008) Deviations : None F9600 technical, batch PL13-0385 Purity: 99.2%	0, 750, 2500, 5000, and 10000 ppm (for toxicology and toxicokinetic groups) Equivalent to : Males (M): 0, 57, 182, 359 and 740 mg/kg bw/d Females (F): 0, 61, 193, 379 & 733 mg/kg bw/d	750 ppm (equivalent to 57 mg/kg bw/d males & 61 mg/kg bw/d females) Based on relative liver weight increases > 15% in both sexes, adverse effect on body weight, body weight gain and food consumption in females at the LOAEL of 2500 ppm	There were no deaths or clinical signs of toxicity **10000 ppm** ↓ body weight (F): 18 %** ↓ body weight gain: 59 %** (F) & 14 % (M) ↓ food consumption (F): 41 %** (days 0-7), 17 %** (days 7-14) and 22 %** (days 14-27) ↓ food consumption (M): 20 % (days 0-7)** *Organ weights* ↑ absolute liver weights: 32 %** (F) & 56 %** (M) ↑ relative liver weights: 61 %** (F) & 65.5 %** (M) ↑ relative kidney weights: 14** % (F & M) *Histopathology - liver* Hepatocellular hypertrophy: 5/5 mild (F) & 4/5 mild + 1/5 moderate (M)

Method, Species, test substance	Doses	NOAEL	Main adverse effects
[redacted] (2015a) **Acceptable**		(The applicant proposed a NOAEL of 5000ppm)	*Clinical chemistry* ↑ total protein (9 % F &13 %** M), ↑ albumin (11 % M**), ↑ globulin (12 % M** & 15 %* F),↑ cholesterol (79 %** M & 91 %** F), ↑ BUN (45.5 %** F), ↑ triglyceride (86 %* F) **5000 ppm** ↓ food consumption (F): 23.5 %** (days 0-7) & 17 %* (days 7-21) ↓ food consumption (M): 16 %* (days 0-7) *Organ weights* ↑ absolute liver weight: 19 %* (F) ↑ relative liver weight: 29 %** (F) & 23 %** (M) *Histopathology - liver* Hepatocellular hypertrophy: 1/5 minimal & 4/5 mild (F); 3/5 minimal & 2/5 mild (M) *Clinical chemistry* ↑ cholesterol (43 %* F) **2500 ppm** ↓ food consumption in females: 12 %** (days 0-7) & 11 %* (days 7-14) *Organ weights* ↑ relative liver weight: 17 %** (F), 15.5 %** (M) *Histopathology - liver* Hepatocellular hypertrophy: 4/5 minimal (F) & 3/5 minimal (M) **750 ppm** None relevant.

B.6.3.2.2. *Oral 28-day study in mice*

One study investigating the effects of bixlozone in mice after 28-days oral (dietary) exposure is available.

Study	A 28-Day Oral (Dietary) Toxicity Study of F9600 Technical in CD-1 Mice
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2015b)
Date performed	Jan-Feb 2014
Test facility	[redacted] & [redacted]
Report reference	Study no. [redacted]-105109
Guideline(s)	OECD Guideline 407 (2008)
Deviations from the guideline	There were no deviations of significance
GLP	Yes
Test material	F9600 technical; Batch PL13-0385 Purity 99.2%
Method of analysis	*The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to the lack of explanation of the use of a non-linear calibration.* However, the method is considered sufficient for regulatory purposes.
Study acceptable	Yes

Material and Methods

In a GLP and OECD guideline study, bixlozone technical was administered *ad libitum* in the diet of CD-1 mice (5/sex/group) for 28 consecutive days at doses of 0, 1000, 2000, 4000 and 5000 ppm equivalent to 0, 187, 381, 788 and 985 mg/kg bw/d in males and 0, 289, 554, 984 and 1384 mg/kg bw/d in females.

Results

Survival and clinical observations

There were no treatment-related deaths reported. A test substance-related clinical observation consisting of yellow material on the urogenital area was noted for the 4000 and 5000 ppm group males at the detailed daily physical examinations. These observations were not considered adverse.

Mean body weight and body weight gain (Table B 6.3.2.6)

The most notable effect observed on body weight was a reduction in the overall body weight gain (day 0-28) by > 10 % in males at 2000 and 4000 ppm and in females at 1000, 2000 and 5000 ppm. However, as no clear dose-response or statistical significance was noted, these effects were considered unrelated to treatment, with the possible exception of the decrease observed in the top dose females. There were no treatment-related effects on food consumption noted in this study.

Overall, treatment-related and adverse body weight gain reduction > 10 % was observed in females at the top dose.

Table B 6.3.2.6: Body weight development in the 28-day mouse study with bixlozone

	Males					Females				
Dose (ppm)	**0**	**1000**	**2000**	**4000**	**5000**	**0**	**1000**	**2000**	**4000**	**5000**
mg/kg bw/day	**0**	**187**	**381**	**788**	**985**	**0**	**289**	**554**	**984**	**1384**
Body weight										
Day 0	30.5 ± 2.80	30.8 ± 1.22	31.0 ± 1.75	31.4 ± 2.15	30.5 ± 2.20	23.5 ± 1.39	23.8 ± 1.74	22.7 ± 2.18	22.8 ± 1.53	22.4 ± 1.69
Day 28	34.1 ± 2.59	34.5 ± 2.36	34.2 ± 1.10	34.4 ± 2.42	34.6 ± 3.70	28.8 ± 2.14	28.5 ± 1.80	27.0 ± 1.66	28.1 ± 1.66	26.8 ± 1.37
% difference from control at day 28	-	+ 1.2	+0.3	+0.9	+1.5	-	-1	-6	-2.4	-7
Overall body-weight gain day 0-28 (g)	3.6 ± 0.82	3.6 ± 1.87	3.2 ± 1.13	3.0 ± 0.73	4.1 ± 1.58	5.3 ± 1.80	4.7 ± 1.24	4.4 ± 1.59	5.3 ± 1.88	4.3 ± 1.07
% difference from control			-11	-17	+14		-11	-17	0	-19

Clinical haematology and chemistry

There were no effects on haematology.

With regards to clinical-chemistry, the only notable change was observed in top dose males at day 28 with a statistically significant higher mean alanine aminotransferase (ALT) value (2.4-fold compared to mean control); however mean ALT values were comparable to controls at lower doses. There were no other test substance-related effects on serum chemistry parameters in both sexes.

Overall a treatment-related and adverse increase in ALT was seen in males only at the top-dose.

Organ changes (Table B 6.3.2.7)

Consistently with the effects observed in the 7-day palatability study in mice ([redacted], 2015c), statistically significant higher liver weights were noted in mice. The mean relative liver weight (to body weight) was increased at the top dose of 5000 ppm by 13 % in males. In females, the mean relative liver weight was statistically significantly increased from 2000 ppm in a dose-dependent manner (+ 11, 21.5 and 24 % at 2000, 4000 and 5000 ppm respectively and compared to controls). These effects are considered treatment-related and adverse from 4000 ppm in females and at the top dose in males (due to associated increase in ALT).

Lower mean relative (but not absolute) heart weights were observed in males only at 4000 and 5000 ppm (-18 and -14.5 % respectively) but no clear dose-response was evident; hence these results are unlikely to indicate an effect of bixlozone on the heart, especially considering that no associated histopathology was seen.

There were no other notable effects seen on any other organ in both sexes. Overall clear treatment-related and adverse increases in liver weight were noted in females from 4000 ppm and in males at the top-dose.

Table B 6.3.2.7: Selected organ weights in the oral 28-day mouse study

	Males					Females				
Dose (ppm)	**0**	**1000**	**2000**	**4000**	**5000**	**0**	**1000**	**2000**	**4000**	**5000**
mg/kg bw/day	**0**	**187**	**381**	**788**	**985**	**0**	**289**	**554**	**984**	**1384**
Terminal body weight (bw)										
(g)	34.1 ± 2.59	34.5 ± 2.36	34.2 ± 1.10	34.4 ± 2.42	34.6 ± 3.70	28.8 ± 2.14	28.5 ± 1.80	27.0 ± 1.66	28.1 ± 1.66	26.8 ± 1.37
Liver weight										
Absolute (g)	2.04 ± 0.23	1.99 ± 0.31	2.03 ± 0.13	2.1 ± 0.25	2.33 ± 0.20	1.57 ± 0.14	1.60 ± 0.13	1.64 ± 0.19	**1.85 ± 0.14**	1.80 ± 0.16
% change from controls	-	-2.5	-0.6	3	14	-	2	5	**18***	15
Relative to bw (g)	5.89 ± 0.46	5.75 ± 0.54	5.94 ± 0.28	6.09 ± 0.40	**6.76 ± 0.53**	5.89 ± 0.46	5.89 ± 0.46	5.89 ± 0.46	5.89 ± 0.46	5.89 ± 0.46
% change from controls	-	-4	-0.7	2	**13***	-	4	**11***	**21.5****	**24****
Heart weight										
Absolute (g)	0.217 ± 0.0279	0.193 ± 0.027	0.195 ± 0.017	0.178 ± 0.017	0.188 ± 0.021	0.169 ± 0.015	0.156 ± 0.023	0.166 ± 0.0012	0.1604 ± 0.0048	0.165 ± 0.023
% change from controls	-	-11	-10	-18	-13	-	-8	-2	-5	-2
Relative to bw (g)	0.635 ± 0.056	0.560 ± 0.055	0.570 ± 0.035	0.520 ± 0.051	0.543 ± 0.028	0.589 ± 0.059	0.546 ± 0.047	0.614 ± 0.012	0.573 ± 0.036	0.616 ± 0.068
% change from controls	-	-12	-10	**-18****	**-14.5***	-	-7	4	-3	**5**

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test

Microscopic findings (Table B 6.3.2.8)

Associated with the increased liver weights observed above, hepatocellular hypertrophy (minimal to mild in severity) was noted from 2000 ppm in females and from 4000 ppm in males. Consistently with liver effects observed in the rat, the microscopic liver findings were characterised by a pale and eosinophilic cytoplasm of a predominantly centrilobular distribution. The incidences in hepatocellular hypertrophy and severity tended to correlate with the changes in liver weights in both sexes.

Table B 6.3.2.8: Microscopic findings in the oral 28-day mouse study

	Males					Females				
Dosage (ppm)	**0**	**1000**	**2000**	**4000**	**5000**	**0**	**1000**	**2000**	**4000**	**5000**
mg/kg bw/day	**0**	**187**	**381**	**788**	**985**	**0**	**289**	**554**	**984**	**1384**
	Histopathology									
Liver (N)	5	5	5	5	5	5	5	5	5	5
Hypertrophy, Hepatocellular	0	0	0	1	4	0	0	2	2	3
Minimal	-	-	-	1	2	-	-	2	2	2
Mild	-	-	-	0	2	-	-	0	0	1

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test
N number of animals examined

Conclusion

In conclusion, in a GLP and OECD guideline study, 28-day dietary administration of bixlozone to mice affected the body weight gains of females at the top dose. Adverse liver findings were seen in females from 4000 ppm (> 15% weight and associated hypertrophy) and in males at the top dose (hypertrophy and increased ALT).

HSE has identified a NOAEL of 2000 ppm (equivalent to 381 mg/kg bw/day in males and 554 mg/kg bw/day in females) from this study, based on adverse liver changes recorded in females from 4000 ppm (788/984 mg/kg bw/day in M/F) (statistically significant increase > 15% of relative liver weight associated with hepatocellular hypertrophy). The applicant proposed a NOAEL of 5000 ppm based on absence of adverse findings.

[redacted] (2015b)

Table B 6.3.2.9: Summary of the 28-day dietary study in the mouse

Method, Species, test substance	Doses	NOAEL	Main adverse effects
28 day, dietary Mouse, Crl:CD-1, males & females, 5/sex/group GLP OECD 407 (2008) Deviations: none F9600 Technical, batch PL13-0385 Purity: 99.2% [redacted] (2015b) **Acceptable**	0, 1000, 2000, 4000, and 5000 ppm Equivalent to: Males: 0, 187, 381, 788 & 985 mg/kg bw/day Females: 0, 289, 554, 984 & 1384 mg/kg bw/day	2000 ppm (554 mg/kg bw/day females) Based on relative liver weight increases > 15 % and hepatocellular hypertrophy from 4000 ppm in females (18.3 % absolute and 21.5% relative) (The applicant proposed a NOAEL of 5000ppm based on absence of adverse findings)	There were no treatment-related deaths. **5000 ppm** ↓ body weight gain: 19 % (F) *Organ weights* ↑ absolute liver weight: 15 % (F) & 14 % (M) ↑ relative liver weight: 24 %** (F) & 13 %* (M) *Histopathology - liver* Hepatocellular hypertrophy: 3/5 F (2 minimal, 1 mild) & 4/5 M (2 minimal, 2 mild) *Clinical chemistry* ↑ ALT: 137 %* (M) **4000 ppm** *Organ weights* ↑ absolute liver weight: 18 %* (F) ↑ relative liver weight: 21.5 %** (F) *Histopathology - liver* Hepatocellular hypertrophy: 2/5 F (minimal) & 1/5 M (minimal) **2000 ppm & 1000ppm** No adverse effects observed

B.6.3.2.3. *Oral 28-day study in dogs*

This dose-range finding GLP dietary study was conducted to determine the appropriate dosage levels for the subsequent 90-day dog study and as such it only broadly follows the OECD guideline for the conduction of 90-day studies (OECD Guideline 409). This is however acceptable since there is no specific guideline for a 28-day repeated dose toxicity study available for non-rodents; the study remains nevertheless informative and is thus reported below.

Study	A 28-Day Oral (Dietary) Toxicity Study of F9600 Technical in Beagle Dogs.
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016b)
Date performed	April 2014
Test facility	[redacted] & [redacted]
Report reference	Study no. [redacted]-105117
Guideline(s)	OECD Guideline 409 (1998)
Deviations from the guideline	Since this is a dose-range finding study aiming to determine the appropriate dosage levels for the 90-day dog study, fewer animals per group were used. Due to palatability issue at 30000 ppm animals were fed with food supplementation thus the mean achieved bixlozone consumption could not be calculated accurately.
GLP	Yes
Test material	F9600 Technical; batch PL14-0049 Purity 96 %,
Method of analysis	*The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to the lack of explanation of the use of a non-linear calibration.* However, the method is considered sufficient for regulatory purposes.
Study acceptable	Yes but as supplementary information only since it is a range-finding study which used a low number of animals per group. In addition the method of administration (diet) led to palatability issues and severe toxicity unrelated to the test substance itself.

Material and methods

Bixlozone technical was administered in the diet of Beagle dogs (2/sex/group) for 28 consecutive days at doses of 0, 1000, 3000, 10000 and 30000 ppm. The concurrent control group was offered the basal diet on a comparable regimen.

Results

Test item consumption and palatability

The mean achieved bixlozone technical consumption in the control, 1000, 3000, and 10000 ppm groups were equivalent to 0, 38, 134 and 370 mg/kg bw/day for males, respectively, and 0, 39, 108, and 309 mg/kg bw/day for females, respectively.

Test substance-related effects (palatability) on food consumption and weight loss were noted at 30000 ppm in both sexes during the first 2 weeks of the study when compared to the control group and acclimation values, prompting the addition of food supplementation with 400 g of wet food for the last 2 weeks of the study to maintain the health of the animals. Therefore, the mean achieved bixlozone consumption for this high-dose group of 30000 ppm could not be calculated accurately.

Clinical observations

There were no deaths. One male in the 30000 ppm group presented a thin body condition and both males at this dose suffered from decreased defecation at various time-points throughout the study; there were no other clinical signs of toxicity.

Body weight changes (Table B 6.3.2.10)

Palatability issues with the test substance at 30000 ppm resulted in lower food consumption and weight loss in both sexes at the start of the study; although subsequent supplementation with wet food after 2 weeks meant that mean body-weight gain for these animals was comparable with controls for the remainder of the study, it still resulted in final mean body weights 17 % and 9 % lower than controls in males and females respectively and overall decreases in body weight gains of 116% and 90% respectively. At 10000 ppm food consumption and

body weight gain of males and females were also lower throughout the study when compared with controls (and the preceding acclimatisation phase). At lower doses, the body weight gain of females was substantially lower from 1000 ppm than controls.

Overall, adverse effects on body weight gain were seen in females from 1000 ppm and from 10000 in males and adverse effects on body weight were noted at the top dose in both sexes due to palatability effects, as seen with food consumption adversely affected in both sexes from 10000 ppm.

Table B 6.3.2.10: Summary of mean body weights and body weight gain in the 28-day dog study

	Males (n = 2)					Females (n = 2)				
Dosage (ppm)	**0**	**1000**	**3000**	**10000**	**30000**	**0**	**1000**	**3000**	**10000**	**30000**
(mg/kg bw/d)	**0**	**38**	**134**	**370**	**1015***	**0**	**39**	**108**	**309**	**1110***
Body weights (kg)										
Day 27	8.3	8.1	8.5	8.2	6.9	7.9	7.2	7.6	7.5	7.2
% difference from control	-	-2.4	2.4	-1.2	-17	-	-9	-4	-5	-9
Body-weight gain (kg)										
Days 0-27	1.2	1.1	1.4	1.0	-0.2	1.1	0.6	0.6	0.5	0.1
% difference from control	-	-	-	-17	-116	-	-45	-45	-54	-90
Food consumption (g/animal/day)										
Days 0-7	350 ± 65.1	219 ± 60.8	361 ± 55.2	252 ± 4.9	35 ± 14.8	286 ± 30.4	270 ± 50.9	244 ± 24.0	191 ± 2.8	160 ± 71.4
Days 7-14	334 ± 47.4	295 ± 19.8	379 ± 20.5	306 ± 14.8	197 ± 82.0	308 ± 5.7	267 ± 12.0	254 ± 25.5	264 ± 27.6	238 ± 125.9
Days 14-21	373 ± 35.4	329 ± 56.6	366 ± 36.8	303 ± 0.0	299 ± 224.2	318 ± 17.7	279 ± 6.4	287 ± 12.0	229 ± 17.7	326 ± 65.1
Days 21-27	324 ± 103.9	297 ± 64.3	298 ± 26.9	287 ± 7.8	373 ± 130.1	309 ± 13.4	253 ± 3.5	256 ± 22.6	206 ± 33.9	297 ± 51.6

* N/A = The mean achieved bixlozone consumption for this high-dose group could not be calculated accurately due to severe palatability effects

Haematology, clinical chemistry and urinalysis (Table B 6.3.2.11)

There were no effects on haematology.

Some changes in clinical chemistry measurements were noted comprising higher cholesterol levels in males from 3000 ppm and in females at 1000 and 30000 ppm compared with controls but without a clear dose-response. Lower values were seen for blood urea nitrogen (BUN) in all treated male groups and from 10000 ppm in females and for serum phosphorous in all treated female groups and from 3000 ppm in males; the applicant highlighted these findings to be a common observation in fasting dogs.

There were no biologically relevant urinalysis findings; an overall higher urinary output in the high-dose females was attributed to one female only and the cause could not be identified.

Overall, there were no adverse effects on haematology, clinical-chemistry and urinalysis.

Table B 6.3.2.11: Summary of clinical chemistry findings in the 28-day dog study

	Males (n = 2)					Females (n = 2)				
Dosage (ppm)	**0**	**1000**	**3000**	**10000**	**30000**	**0**	**1000**	**3000**	**10000**	**30000**
(mg/kg bw/d)	**0**	**38**	**134**	**370**	**1015***	**0**	**39**	**108**	**309**	**1110***
Clinical chemistry										
Cholesterol (mg/dL)										
Day -4	164	159	183	225	145	142	147	142	153	168
Day 28	18	164	210	265	208	147	165	147	191	225
% difference from control (day 28)	-	-12	+13	+42.5	+12	-	+12	0	+30	+53
Phosphorus (mg/dL)										
Day -4	6.9	7.1	6.7	6.8	7.1	7.0	6.5	6.3	6.4	6.9
Day 28	6.6	6.6	6.4	6.0	5.5	6.9	5.7	5.7	5.6	5.9
% difference from control (day 28)	-	0	-3	-9	-17	-	-17	-17	-19	-14.5
BUN (mg/dL)										
Day -4	13.1	9.3	10.3	12.1	11.3	12.1	14.6	17.2	13.1	12.5
Day 28	15.3	12.2	11.7	12.4	9.1	14.9	14.4	16.7	12.2	11.5
% difference from control (day 28)	-	-20	-23.5	-19	-40.5	-	-3	+12	-18	-23

* N/A = The mean achieved bixlozone consumption for this high-dose group could not be calculated accurately due to severe palatability effects

Organ weight changes (

Table B 6.3.2.12*)*

Consistently with the findings in the rat and mouse, there were notable changes in the liver. Mean relative liver weights (to body weights) were increased by > 15 % from 10000 ppm in both sexes; the changes are considered treatment-related and adverse.

Kidney weights in the top-dose group were greater than in controls in females (+ 28 % absolute and + 4 % relative) and in males (+ 20 % absolute and + 41 % relative); absolute and relative kidney weights were also increased at 10000 ppm in males only (+ 22 % absolute and + 23 % relative). Histopathological findings were observed at top-dose in both sexes.

The relative weights of the adrenal glands were increased at 30000 pm (+ 31 % and + 23 % in males and females respectively) but a clear dose-response was not evident. Lower mean thymus weights were also seen in males at this dose (-30 %). Furthermore, lower prostate weights were observed from 3000 ppm; however according to the histopathological observations, the changes correlated with marked immature sexual development in one 10000 ppm and one 30000 ppm male (similar but less marked signs of immaturity were noted in all males at these doses).

In females, variable ovary and uterus/cervix weights changes were observed; however, they did not show a clear dose-response and were unaccompanied by any histopathological correlates. These changes are thus not considered treatment-related.

Overall treatment-related and adverse effects on the liver were observed in both sexes from 10000 ppm and on the kidney from 10000 ppm in males and at top-dose in females. In addition, adrenal weights were increased at the top dose in both sexes, thymus weights were decreased in top dose males and prostate weights were decreased from 3000 ppm.

Table B 6.3.2.12: Selected organ weight changes in the 28-day dog study

	Males (N = 2)					Females (N = 2)				
Dosage (ppm)	**0**	**1000**	**3000**	**10000**	**30000**	**0**	**1000**	**3000**	**10000**	**30000**
(mg/kg bw/d)	**0**	**38**	**134**	**370**	**1015***	**0**	**39**	**108**	**309**	**1110***
	Organ weights									
Terminal bodyweight (kg)	8.1 ± 0.3	7.9 ± 0.9	8.2 ± 0.8	8.1 ± 0.2	6.9 ± 1.0	7.5 ± 0.9	6.9 ± 0.5	7.2 ± 0.6	7.1 ± 0.7	6.8 ± 0.7
Liver (g)	233.3 ± 15.73	206.9 ± 1.41	246.35 ± 41.57	278.3 ± 11.62	303.9 ± 46.245	190.2 ± 48.92	188.49 ± 1.71	209.29 ± 18.095	230.05 ± 5.996	311.05 ± 53.005
% difference from control	-	-	-	+19	+30	-	-	-	+21	+63.5
R	-	-8.0	4.3	20	53		8.8	15	28.5	80
Kidney (g)	38.9 ± 0.15	44.04 ± 2.38	44.5 ± 3.67	47.5 ± 6.70	46.7 ± 8.78	38.1 ± 3.66	35.51± 1.37	36.04 ± 3.345	35.8 ± 3.72	48.6 ± 0.27
% difference from control	-	+13	+14	+22	+20	-	-	-	-	+28
R		17	13.5	23	41		1.2	-2.5	-1.6	40
Adrenal gland (g)	1.079 ± 0.0037	1.0445 ± 0.1136	0.912 ± 0.182	0.985 ± 0.1325	1.165 ± 0.099	0.988 ± 0.2468	0.864 ± 0.0146	1.138 ± 0.0608	1.054 ± 0.1519	1.10 ± 0.077
% difference from control	-	-3.2	-15	-8.7	+8	-	-13	+15	+6.7	+11
R	-	0	-15	-7.7	+31	-	0	+23	+15	+23
Thymus (g)	5.86 ± 0.51	8.39 ± 0.48	7.64 ± 3.32	5.86 ± 0.46	3.72 ± 2.98	6.19 ± 1.00	7.83 ± 2.75	6.96 ± 2.27	7.02 ± 1.655	6.74 ± 1.19
% difference from control	-	+43	+30	0	-36.5	-	+26.5	+12	+13	+8.9
R		+49	+28	+1.4	-29		+40	+16	+22	+19
Prostate (g)	1.57 ± 0.742	1.80 ± 0.721	1.09 ± 0.021	1.09 ± 0.438	0.87 ± 0.304	-	-	-	-	-
% difference from control	-	14.6%	-30.6%	-30.6%	-44.6%	-	-	-	-	-
R		21.1	-31.6	-26.3	-36.8	-	-	-	-	-
Uterus/cervix (g)	-					2.17 ± 0.028	1.55 ± 0.262	1.85 ± 1.004	2.02 ± 0.537	0.81 ± 0.184
% difference from control			-			-	-28.6%	-14.7%	-6.9%	-61.3%
R							-20.7	-10.3	-3.4	-58.6

* N/A = The mean achieved bixlozone consumption for this high-dose group could not be calculated accurately due to severe palatability effects R % change in relative weight (organ weight to bodyweight) from control
N Number of animals examined

Microscopic findings (Table B 6.3.2.13)

There were histopathological findings in the liver and kidneys which correlated to the weight changes observed in these organs. In the liver minimal to mild hepatocellular hypertrophy was noted from 10000 ppm in both sexes, characterised by enlarged hepatocytes with distended eosinophilic cytoplasm which obliterated the sinusoidal spaces. The absence of correlating biochemical findings in levels of circulating ALP, AST, ALT, GGT or SDH indicates that these histopathological findings are compatible with an adaptive response to treatment.

In the kidney, minimal to mild interstitial inflammation and renal tubular hypertrophy (mild to moderate) was seen in both sexes of the top dose group; there were no correlating haematology, chemistry or urinalysis findings but increased organ weights were observed at top-dose in both sexes.

Minimal to mild single cell necrosis was observed in the pancreas in one male at each dose of 3000, 10000, 30000 ppm. Also, immature prostate was noted from 10000 ppm.

Lymphoid depletion and/or single cell necrosis was observed in the thymus in males at 10000 and 30000 ppm.

Overall there were histopathological findings in the liver from 10000 ppm in both sexes, in the kidney at the top dose in both sexes, in the thymus from 10000 ppm in males, in the pancreas from 3000 ppm in males and in the prostate from 10000 ppm.

Table B 6.3.2.13: Selected microscopic findings from the 28-day dog study

	Males (N = 2)					Females (N = 2)				
Dosage (ppm)	**0**	**1000**	**3000**	**10000**	**30000**	**0**	**1000**	**3000**	**10000**	**30000**
(mg/kg bw/d)	**0**	**38**	**134**	**370**	**1015***	**0**	**39**	**108**	**309**	**1110***
	Microscopic findings									
Liver										
Hypertrophy, hepatocellular, diffuse	0	0	0	2	2	0	0	0	2	2
Minimal	-	-	-	2	1	-	-	-	2	0
Mild	-	-	-	0	1	-	-	-	0	2
Kidney										
Hypertrophy, renal tubular epithelium	0	0	0	1	1	0	0	0	0	0
Mild	-	-	-	0	1	-	-	-	-	-
Moderate	-	-	-	1	0	-	-	-	-	-
Inflammation, interstitial	0	0	0	0	1	0	0	0	0	2
Minimal	-	-	-	-	1	-	-	-	-	1
Mild	-	-	-	-	0	-	-	-	-	1
Prostate										
Immature (juvenile development)	0	0	0	1	1					
Pancreas										
Necrosis, minimal	-	-	1	1	-	0	0	0	0	0
Necrosis, mild	-	-	-	-	1	0	0	0	0	0
Thymus										
Lymphoid depletion, mild	-	-	-	1	1	0	0	0	0	0
Lymphoid depletion, moderate	-	-	-	-	1	0	0	0	0	0
Single cell necrosis, minimal	-	-	-	1	-	0	0	0	0	0

* N/A = The mean achieved bixlozone consumption for this high-dose group could not be calculated accurately due to severe palatability effects
N Number of animals examined

Conclusion

In this range-finding study, dietary administration of bixlozone technical to Beagle dogs for 28 days caused palatability issues at the top-dose of 30 000 ppm, resulting in severely reduced food consumption and overall lower body weights and body weight gains; food supplementation to the top dose animals resolved the palatability issues, but bodyweight gain of this dose group at the end of the study was still markedly lower than controls. Adverse effects on body weight gain were seen in females from the lowest dose of 1000 ppm and in males from 10000 ppm. The liver and kidney were the main target organs of toxicity. Adverse (> 15%) increases in liver weight with associated hypertrophy were seen in both sexes from 10000 ppm. Kidney weights were increased from 10000 ppm in males and at the top dose in females. Associated histopathology occurred at the top dose in both sexes. In addition, adrenal weights were increased in both sexes at the top dose, thymus histopathology occurred in males from 10000 ppm, prostate weight was decreased from 3000 ppm with associated histopathology from 10000 ppm and histopathological findings (necrosis) were observed in the pancreas from 3000 ppm in males. Based on these findings, a NOAEL cannot be identified from this study as

effects on body weight gain were seen in females from the lowest dose tested. A LOAEL of 1000 ppm (39 mg/kg bw/d) was established. However, as this was a range-finding study which used only 2 dogs/sex/group, and the method of administration (diet) led to palatability issues and severe toxicity unrelated to the test substance itself, this LOAEL is not robust.

[redacted] (2016b)

Table B 6.3.2.14: Summary of the 28-day dietary study in the dog

Method, Species, test substance	Doses	NOAEL	Main adverse effects
28 day, dietary Dog, Beagle, males & females, 2/sex/group Bixlozone technical, batch PL14-0049 Purity: 96% Vehicle: acetone GLP Dose-range finding study (loosely follows OECD 409) [redacted] (2016b) **Supplementary only**	0, 1000, 3000, 10000 & 30000 ppm Equivalent to control, 1000, 3000, and 10000 ppm groups: Males: 0, 38, 134 & 370 mg/kg bw/day Females: 0, 39, 108 & 309 mg/kg bw/day (test substance intake for 30000 ppm males and females could not be accurately calculated due to food supplementation)	No robust NOAEL or LOAEL could be set from this study as effects on body weight gain were seen in females from the lowest dose tested (due to method of administration, palatability issues and severe toxicity) and this is a range-finding study. LOAEL = 1000 ppm (39 mg/kg bw/day)	There were no treatment related deaths No statistical analysis was performed **30000 ppm** Clinical signs: thin body condition (1 M), ↓ defecation (2 M) ↓ body weight: 17 % (M) and 9 % (F) ↓ body-weight gain: 116 % (M) and 90 % (F) ↓ food consumption led to food supplementation (M & F) ↑ relative liver weight: 80 % (F) and 53 % (M) ↑ absolute liver weight: 30 % (M) and 63.5 % (F) ↑ relative kidney: 41 % (M) and 40 % (F) ↑ absolute kidney weight: 20 % (M) and 28 % (F) Hepatocellular hypertrophy in 2 / 2 M (1 minimal & 1 mild) Hepatocellular hypertrophy in 2/ 2 F (mild) **10000 ppm** ↓ body-weight gain: 17 % (M) and 54 % (F) ↓ food consumption in M & F ↑ Relative liver weight: 28.5 % (F) and 20 % (M) ↑ absolute liver weight: 19 % (M) and 21 % (F) ↑ kidney weight in M: 22 % absolute and 23 % relative Hepatocellular hypertrophy in 2 / 2 M (minimal) Hepatocellular hypertrophy in 2 / 2 F (minimal) **3000 ppm** ↓ body weight gain: 45.5 % (F) ↑ relative liver weight: 15 % (F) **1000 ppm** ↓ body weight gain: 45.5 % (F)

B.6.3.3. Oral 90- day studies

The repeated-dose toxicity of bixlozone has been investigated after 90-days' oral exposure in rats, mice and dogs.

B.6.3.3.1. *Oral 90-day study in rats*

One study investigating the repeated-dose toxicity of bixlozone in rats after oral administration for 90-days is available. The study includes a dedicated neurotoxicity phase as well as a 28-day recovery phase following 90-day exposure. The neurotoxicity assessment is reported in the Section B.6.7 of the DAR.

Study	A 90-Day Dietary Combined Toxicity and Neurotoxicity Study of F9600 in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted](2016a)
Date performed	March-August 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105119
Guideline(s)	OECD 408 and 424
Deviations from the guideline	There were no deviations of significance
GLP	Yes (lab certified by National Authority)
Test material	F9600 Technical; batch PL14-0049 Purity 96%,
Method of analysis	*The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to the lack of explanation of the use of a non-linear calibration.* However, the method is considered sufficient for regulatory purposes.
Study acceptable	Yes

Material and Methods

In a GLP and OECD guideline study, bixlozone technical was administered via the diet to Sprague Dawley rats for 90 consecutive days. Dietary concentrations were 0, 500, 2000 and 8000 ppm in males (equivalent to 0, 29, 121 and 505 mg/kg bw/day) and 0, 500, 2000 and 5000 ppm in females (equivalent to 0, 37, 150 and 351 mg/kg bw/day). The dietary levels were selected on the results of a previous 28-day dietary study ([redacted], 2015a) where females displayed more severe effects than males on body weight, body weight gain and food consumption at 10000 ppm. Therefore, the highest dose levels of 8000 and 5000 ppm were selected for the males and females, respectively, for the current study to show signs of toxicity including increased liver weights and decreases in body weight gain without producing mortality. Low and mid-dose levels of 500 and 2000 ppm, respectively, were chosen to investigate the dose response of the test substance.

The control and high-dose groups comprised 21 rats/sex/group whilst the low- and mid-dose groups comprised 16 rats/sex/group. Of these animals 10 /sex/group were randomly assigned for the functional observational battery (FOB) and motor activity assessments, with six of these being assigned to a dedicated neurotoxicity phase; the remaining rats were assigned to the general toxicity phase. Ten rats/sex/group were euthanised after 90-days' administration of the test substance and the five remaining animals in the control and high-dose groups were euthanized after a further 28-day recovery period.

Results

Clinical observations and survival

One male of the 8000 ppm group was found dead on day 87; the cause of death could not be determined. There were no treatment-related signs of toxicity in any animal. In the absence of further information, this death at the top dose is considered treatment-related.

Body weight changes (Table B 6.3.3.1)

Statistically significant lower mean body-weight gains were noted for both sexes of the high-dose group sporadically throughout the study, which culminated in body weight gains at the end of the study (for days 0-90) that were -18 % and -23 % lower than controls for males and females respectively. This corresponded to mean body weights that were approximately 9 % lower than the corresponding controls. During the recovery period (days 91-118) the mean body weight gains for males at 8000 ppm and females at 5000 ppm were notably increased compared to control animals to reach a final body weight generally similar to the control groups.

Overall treatment-related and adverse lower mean body weights and overall body weight gains (day 0-90 days) were observed at the top-dose (8000 ppm in males and 5000 ppm in females) in both sexes; the effect appeared to be reversible during a recovery period of 28 days.

Table B 6.3.3.1: Summary of body weight changes in the 90-day rat study

	Males				Females			
Dosage (ppm)	**0**	**500**	**2000**	**8000**	**0**	**500**	**2000**	**5000**
mg/kg bw/d	**0**	**29**	**121**	**505**	**0**	**37**	**150**	**351**
Final bodyweight (g) Day 90	569 ± 58.8	584 ± 59.6	569 ± 53.9	**517* ± 50.7**	294 ± 23.3	301 ± 37.0	286 ± 25.8	**266** ± 21.8**
% difference from controls	-	+ 2.6	0	- **9.1**	-	+ 2.4	- 2.7	- **9.5**
Body weight gain (g) (days 0-90)	289 ± 42.7	298 ± 43.0	284 ± 36.3	**238** ± 33.8**	114 ± 19.1	124 ± 20.4	110 ± 19.8	**88** ± 11.9**
% difference from controls	-	+ 3.1	- 1.7	- **18**	-	+ 8.8	- 3.5	- **23**
Final recovery body weight Day 118	568 ± 97.6			560 ± 37.6	305 ± 27.6			293 ± 24.9
% difference from controls	-			-1.4	-			-3.9
Recovery body weight gain (g) (days 91-118)	35 ± 10.9			46 ± 8.1	17 ± 9.0			27 ± 10.1
% difference from controls	-			+31				+59

Food consumption and food utilisation efficiency ***(Error! Reference source not found.)***

In males a statistically significant reduction in food consumption and food utilisation efficiency (equivalent to body weight gain as percent of food consumed) was observed during the first week of dosing at 8000 ppm. Even though food consumption in males recovered to be similar to controls throughout the remainder of the study, the overall food utilisation efficiency remained statistically significantly lower compared to controls (- 14 %). Since these results correlate with an overall lower mean body weight gain in this dose-group, they are considered to be test substance-related. During the recovery period the food consumption and food efficiency increased for the top-dose males compared to the controls, showing that the effects seen during treatment were reversible.

In females statistically significantly lower mean food consumption was noted for days 0-90 at the high-dose only, to the extent of -8 % (difference from controls). This corresponded to a lower food utilisation efficiency of -11 % compared with controls for the duration of the study. The lower food consumption and food utilisation efficiency correlated with lower mean body weight gains throughout the study period and HSE considers this to be a treatment-related effect. During the recovery period the food consumption and food efficiency increased for the top-dose females compared to the controls, showing that the effects seen during treatment were reversible.

Overall adverse effects on food consumption and food utilisation efficiency were seen in both sexes at the top dose. In males, the decreases in food consumption were seen mainly during the first week of treatment. The effects were reversible by the end of the recovery period.

Table B 6.3.3.2: Food consumption and food efficiency in the 90-day rat study including the recovery period.

	Males				Females			
Dosage (ppm)	**0**	**500**	**2000**	**8000**	**0**	**500**	**2000**	**5000**
mg/kg bw/d	**0**	**29**	**121**	**505**	**0**	**37**	**150**	**351**
	Main study (day 0-90)							
Food consumption (g/kg/day) (days 0 – 90)	60 ± 2.0	59 ± 1.8	60 ± 2.9	**63** ± 3.2**	76 ± 5.4	75 ± 6.2	75 ± 5.3	**70** ± 4.7**
% difference from controls	-	- 1.7%	0	**+ 5**	-	- 1.3	- 1.3	**-8**
Body weight gain as percent of food consumed (days 0 – 90)	11.5 ± 1.08	11.7 ± 0.91	11.1 ± 0.86	**9.9** ± 0.73**	6.5 ± 0.88	7.2* ± 0.73	6.5 ± 0.66	**5.8* ± 0.68**
% difference from controls	-	+1.7	-3.5	**-14**	-	+11	0	**-11**
	Recovery period (day 91-118)							
Food consumption (g/kg/day)	46 ± 2.2	N/A	N/A	**51** ± 2.3**	66 ± 10.4	N/A	N/A	69 ± 5.7
% difference from controls	-	N/A	N/A	**+ 11**	N/A	N/A	N/A	+ 4.5
Body weight gain as percent of food consumed	5.1 ± 1.24			6.2 ± 0.87	3.3 ± 1.88			5.1 ± 1.69
% difference from controls	-			**+22**	-			**+55**

Haematological parameters, serum chemistry and urinalysis (Table B 6.3.3.3)

There was no effect on haematological parameters; however, some treatment-related alterations in serum chemistry were noted.

As observed in the 28-day rat study, there were changes in serum cholesterol in males (77 % greater than controls) which were linked on an individual animal basis to vacuolation in the liver (please refer to microscopic findings further below). A statistically significant increase in cholesterol levels was also seen in females in the mid- and high-dose groups. Even though there was no clear dose-response in females, these changes are considered to be treatment-related and adverse. It is also noted that cholesterol levels remained high in females at top-dose following 28 days of recovery.

With regard to serum protein parameters there was an increase in serum globulin in females at 2000 and 5000 ppm (+ 11 % for each dose); however the levels returned for the top-dose group to values similar to the control group by the end of the recovery period. Increased globulin was also observed in the 28-day rat study ([redacted] (2015a)). These effects are considered to be adverse.

There was statistically significantly higher mean serum calcium (measured as bound serum calcium) in the 2000 and 5000 ppm female groups in comparison to controls. These changes were associated with an increased (but not statistically significant) increase in albumin, the main major serum binding protein for calcium, for these female groups. Higher serum calcium associated with higher serum albumin was also reported in the 28-day study in the rat. Chloride concentration was statistically lower in males only at the top-dose compared to controls. These effects are considered treatment-related and adverse.

In regard to the parameters indicators of liver function there was a statistically significantly increase in alanine aminotransferase (ALT) in the 8000 ppm males at week 13 compared to controls. Conversely in females a decrease in ALT was observed. Following the recovery period there was no difference between the top-dose group and controls in males whilst for females the ALT value at top-dose was increased compared to controls. Overall no clear pattern can be drawn from these results; hence these changes in ALT are regarded unrelated to treatment.

All other statistically significant clinical chemistry findings were either within the range of the laboratory historical control data, showed no dose-response or were not associated with any histopathological findings.

Urinalysis revealed no unus+11 ual findings.

Overall treatment-related and adverse effects on some clinical-chemistry parameters (cholesterol, globulin, calcium) indicative of potential liver toxicity were seen from 2000 ppm.

Table B 6.3.3.3: Selected clinical chemistry findings from the 90-day rat study

	Males				Females			
Dosage (ppm)	**0**	**500**	**2000**	**8000**	**0**	**500**	**2000**	**5000**
(mg/kg bw/d)	**0**	**29**	**121**	**505**	**0**	**37**	**150**	**351**
Clinical chemistry (Week 13, and Week 17 –end of recovery period)								
Cholesterol (mg/dL)								
Week 13	65 ± 13.4	76 ± 13.7	69 ± 12.3	**115** ± 24.9**	79 ± 9.8	88 ± 18.4	**114** ± 32.7**	**111* ± 24.2**
% from control	-	+17	+6	+77	-	+11	**+44**	**+40.5**
Week 17	72 ± 12.2			71 ± 16.9	72 ± 9.7			**94** ± 7.4**
% from control	-			-	-			**+30**
Globulin (g/dL)								
Week 13	2.8 ± 0.10	2.9 ± 0.29	2.8 ± 0.21	2.9 ± 0.16	2.8 ± 0.21	2.9 ± 0.24	3.1 ± 0.24	**3.1* ± 0.17**
% from control	-	+3.6	0	+3.6	-	+3.6	+11	**+11**
Week 17	3.1 ± 0.29			2.9 ± 0.11	3.0 ± 0.23			3.1 ± 0.11
% from control	-			-6.5	-			**+3**
Calcium (mg/dL)								
Week 13	11.1 ± 0.40	11.3 ± 0.67	11.2 ± 0.43	11.4 ± 0.51	11.2 ± 0.35	11.4 ± 0.37	**11.7* ± 0.36**	**11.7* ± 0.34**
% from control	-	+2	+1	+3	-	+2	**+4.5**	**+4.5**
Week 17	10.3 ± 0.49			10.2 ± 0.45	10.9 ± 0.35			10.9 ± 0.47
% from control	-			-1	-			0
Albumin								
Week 13	4.1 ± 0.19	4.2 ± 0.38	4.1 ± 0.36	4.3 ± 0.30	4.6 ± 0.33	4.7 ± 0.22	4.7 ± 0.43	4.9 ± 0.30
% from control	-	+2.4	-	+4.9	-	+2.2	+2.2	+6.5
Week 17	4.2 ± 0.32	-	-	4.1 ± 0.12	4.7 ± 0.39	-	-	4.9 ± 0.35
% from control		-	-	-2.4				+4
ALT								
Week 13	37 ± 4.5	41 ± 5.8	41 ± 7.8	**48** ± 10.1**	46 ± 15.5	48 ± 21.0	40 ± 17.2	34 ± 6.8
% from control		+11	+11	**+30**		+4	-13	-26
Week 17	41 ± 11.0			41 ± 7.7	43 ± 15.5			84 ± 51.6
% from control				0				+95

Macroscopic examinations

There were no macroscopic or microscopic findings to explain the morbidity or death of the male found dead at day 87 in the 8000 ppm group. The gross necropsy observations revealed no observations that were considered to be associated with administration of the test substance.

Organ weight changes (Table B 6.3.3.4 & Table B 6.3.3.5)

Statistically significant changes in organ weights comprised increased liver and kidney weights in both sexes. At the end of treatment, liver weights in females were higher (> 15%) than controls at 2000 ppm (+ 16 % absolute and + 17 % relative) and at 5000 ppm (+ 22.5 % absolute and + 34 % relative). In males, statistical significance and levels > 15% were only reached at the top-dose (+ 20 % absolute and + 34 % relative). After the 28-day

recovery period the relative liver weights remained greater than those of the controls, reaching statistical significance in males of the top dose (+ 10 %).

Kidney weights were statistically significantly greater than controls in males at 2000 ppm (+ 15 % absolute and + 14.5 % relative) and at 8000 ppm (+ 21.5 % absolute and + 37 % relative), whilst in females the relative kidney weight was statistically significantly higher at the top-dose only (+ 17 % compared to controls). The weight increase was maintained following the recovery period in both sexes at the top dose, reaching statistical significance for males (+ 22 % compared to the corresponding control).

Similar weight increases in the liver and kidneys were observed in rats after 28-days' exposure ([redacted] (2015a)), however the kidney changes appeared to escalate in severity with duration of exposure (from 28 up to 90 days); the extent of the effects observed in the liver appeared to be consistent regardless of the duration of exposure. Nevertheless the magnitude of the liver weight increases observed from 2000 ppm in females and at 8000 ppm in males, the kidney weight increases observed at the top-dose in females and from 2000 ppm in males are considered to be adverse and treatment-related. This is supported by the fact that the weight changes remained pronounced following the recovery period of 28 days.

Absolute and relative uterus weights were increased, in particular at the top dose of 5000 ppm. These increases were not statistically significant and high interindividual variation (high SDs) was noted. In addition, no associated histopathology was observed. It is also noted that similar increases were observed at the top dose of 3000 ppm in the F0 generation of the 2-generation study; however, due to inconsistencies between generations and high inter-individual variability, these findings were also considered to be spurious. Therefore, HSE concludes that these uterus weight changes are chance findings unrelated to treatment.

There were no biologically relevant or statistically significant effects on the weights of any other organ.

Overall treatment-related and adverse increases in liver weight were observed in males at top-dose (8000 ppm) and in females from 2000 ppm; kidney weights were also adversely affected from 2000 ppm in males whilst at top-dose only for the females.

Table B 6.3.3.4: Selected organ weights from the 90-day rat study at week 13 (day 0-90)

	Males				Females			
Dosage (ppm)	**0**	**500**	**2000**	**8000**	**0**	**500**	**2000**	**5000**
mg/kg bw/d	**0**	**29**	**121**	**505**	**0**	**37**	**150**	**351**
	Organ weights (g) at week 13 (day 0-90)							
Final body weight	545 ± 40.9	569 ± 64.1	546 ± 35.4	**484* ± 59.6**	272 ± 21.8	286 ± 40.0	267 ± 24.2	247 ± 23.2
Liver weight	14.75 ± 1.52	15.6 ± 2.46	15.7 ± 1.18	**19.8** ± 3.795**	7.56 ± 0.74	8.62 ± 1.27	**8.80* ± 0.98**	**9.26** ± 0.65**
% difference from controls	-	+6	+6.5	**+ 34**	-	+14	**+16**	**+22.5**
% Relative weight		+1	+6.4	**+51****		+7.6	**+17****	**+34****
Kidney weight	3.49 ± 0.32	3.77 ± 0.33	**4.01* ± 0.37**	**4.24** ± 0.57**	2.04 ± 0.24	2.24 ± 0.24	2.07 ± 0.21	2.14 ± 0.15
% difference from controls	-	+8	**+15**	**+21.5**	-	+10	+1.5	+5
% Relative weight	-	+3.7	**+14.5****	**+37****	-	+5	+3.3	**+17****
Uterus weight					0.59 ± 0.21	0.73 ± 0.31	0.70 ± 0.22	0.92 ± 0.56
% difference from controls					-	+24	+19	+56
% Relative weight					-	+16	+21	**+67**

* $p < 0.05$, ** $p < 0.01$ determined as statistically-significantly different to control using appropriate statistical test
% Relative weight =% change in relative weight (organ weight to bodyweight) from control

Table B 6.3.3.5: Selected organ weights from the 90-day rat study at week 17 (end of recovery period – day 118)

	Males		Females	
Dosage (ppm)	**0**	**8000**	**0**	**5000**
mg/kg bw/d	**0**	**505**	**0**	**351**
Final body weight	537 ± 93	525 ± 37	280 ± 26	270 ± 23
Liver weight	14.22 ± 2.74	15.33* ± 2.06	8.21 ± 0.96	8.92 ± 1.06
% Difference from controls	-	+8	-	+10
% Relative weight	-	10*	-	13
Kidney weight	3.38 ± 0.54	**4.04** ± 0.38**	1.85 ± 0.78	2.08 ± 0.20
% Difference from controls	-	**+19.5**	-	+12.5
% Relative weight	-	**22**	-	20
Uterus weight			1.22 ± 0.44	0.69 ± 0.19
% Difference from controls			-	-43
% Relative weight			-	20

Microscopic examinations (Table B 6.3.3.6)

Hepatocellular hypertrophy was noted in males at 8000 ppm (1/10 minimal, 6/10 mild & 3/10 moderate) and in females at 2000 ppm (1/10 mild) and at 5000 ppm (7/10 mild & 3/10 moderate) and was characterised by enlarged centrilobular hepatocytes with pale, eosinophilic cytoplasm and prominent rough endoplasmic reticulum. Macrovascular vacuolation was noted in 5/10 males at the top dose only (4 minimal and 1 moderate) which persisted after the recovery period in 1/5 males (minimal). The findings correlated with the other relevant liver-related effects highlighted in this study (increased absolute and relative liver weights and changes in some clinical-chemistry parameters) and in the preceding 28-day rat study.

In the thyroid, mild follicular cell hypertrophy was noted in 3/10 males and 5/10 females in the top-dose group only; this was not observed by the end of the recovery period.

There were no other treatment-related microscopic findings in any other organ including the kidney for which clear organ weight changes were observed after 90 days and following the recovery period; no histopathology findings were seen in the uterus.

Overall, histopathological findings were seen in the liver from the mid dose and in the thyroid at the top dose.

Table B 6.3.3.6: Selected microscopic findings from the 90-day rat study

	Males				Females			
Dosage (ppm)	**0**	**500**	**2000**	**8000**	**0**	**500**	**2000**	**5000**
(mg/kg bw/d)	**0**	**29**	**121**	**505**	**0**	**37**	**150**	**351**
Microscopic findings at Week 13								
Liver (N = 10)								
Hypertrophy, Hepatocellular	0	0	0	10	0	0	1	10
Minimal	-	-	-	1	-	-	0	0
Mild	-	-	-	6	-	-	1	7
Moderate	-	-	-	3	-	-	0	3
Vacuolation, macro vesicular	0	0	0	5	0	0	0	0
Minimal	-	-	-	4	-	-	-	-
Moderate	-	-	-	1	-	-	-	-
Thyroid gland (N = 10)								
Follicular cell hypertrophy (mild)	0	0	0	3	0	0	0	5
Microscopic findings at Week 17								
Liver (N = 5)								
Hypertrophy, Hepatocellular	0	0	0	0	0	0	1	0
Vacuolation, macro vesicular	0	0	0	1	0	0	0	0
Minimal	-	-	-	1	-	-	-	-
Thyroid gland (N = 5)								
Follicular cell hypertrophy (mild)	0	0	0	0	0	0	0	0

Neurotoxicity (Table B 6.3.3.7)

A dedicated neurotoxicity phase was performed in this study: the details of the methods used as well as the findings are reported in more details in Section B.6.7. In summary, according to the Functional observation battery (FOB) performed on 10 animals/ sex/group there was no relevant effect on home cage, handling, sensory or neuromuscular parameters with some exceptions that were considered not treatment-related. Motor activity patterns (mean ambulatory and total mean motor activity) were unaffected by treatment.

The dedicated neurotoxicity phase performed on 6 animals/sex/group did not show alterations to brain weight or length; however, there was a statistically significant higher group mean brain width in females at 5000 ppm (top-dose). At this dose level females showed treatment-related systemic adverse effects with lower mean body weights, body weight gains and food consumption. Since this parameter change was not accompanied with any other neuropathological findings in the high-dose groups and compared to the control groups, the UK considers the effect not adverse in nature since it appears to be more likely related to systemic effects rather than the expression of a specific neurotoxic effect.

Central and peripheral nervous tissues were evaluated in the control and high-dose animals and no treatment-related microscopic lesions or other unusual findings were noted.

Table B 6.3.3.7: Summary of brain weights and brain measurements

	Males (n = 6)				Females (n = 6)			
	Dose (mg/kg bw/day)							
	0	**500**	**2000**	**8000**	**0**	**500**	**2000**	**5000**
Brain weight (g)	2.32 ± 0.134	2.24 ± 0.055	2.32 ± 0.078	2.31 ± 0.070	2.08 ± 0.068	2.03 ± 0.086	2.13 ± 0.071	2.12 ± 0.097
% from control		-3.4	0	-0.4		-2.4	+2.4	+1.9
Brain width (mm)	15.88 ± 0.478	15.58 ± 0.319	15.81 ± 0.451	15.79 ± 0.247	15.06 ± 0.162	15.03 ± 0.229	15.33 ± 0.314	**15.47 ± 0.321***
% from control		-1.9	-0.44	-0.57		-0.2	+1.79	**+2.72**

* Significantly different from the control group at 0.05 using Dunnett's test

Conclusion

Consistent with the findings of the 28-day study conducted in rats, the main effects of bixlozone in this guideline 90-day study were seen in the liver and kidneys. Absolute and relative liver weights were statistically significantly increased and > 15 % in both sexes from 2000 ppm in females and at top-dose of 8000 ppm in males; relative liver weights remained greater than controls in both sexes after the recovery period. There were histopathological correlates (hepatocellular hypertrophy) at the top-dose in males and females which were graded from minimal to moderate. Vacuolation was also observed in top dose males. HSE considers the liver effects observed at the top-dose in males and from 2000 ppm in females to be treatment-related and adverse.

The increases in kidney weights at 2000 ppm (+ 15 % absolute and + 14.5 % relative) and 8000 ppm in males (+ 21.5 % absolute, + 36% relative) and at 5000 ppm in females (+ 17 % relative) remained high at the top-dose following recovery and are also considered by HSE to be treatment-related and adverse; there were however no histopathological correlates reported for the kidney.

There was also mild follicular cell hypertrophy in the thyroid at top-dose in both sexes.

There were no relevant signs of neurotoxicity either in the FOB or motor activity assessments or in the histopathological investigations of the nervous system tissues.

Adverse effects on body weights, body weight gains, food consumption and food utilisation efficiency were also seen at the top dose in both sexes, with one death occurring in males.

Based on these findings, a NOAEL of 500 ppm (equivalent to 29 and 37 mg/kg bw/day in males and females respectively) has been set from this study, with a LOAEL of 2000 ppm (121/150 mg/kg bw/day in M/F).

[redacted] (2016a)

Table B 6.3.3.8: Summary of the 90-day dietary study in the rat

Method, Species, test substance	Doses	NOAEL	Main adverse effects
90-day, dietary (Includes neurotoxicity and recovery phase) Rat, Crl :CD9(SD), males & females, 21/sex/group or 16/sex group (including neurotoxicity phase) F9600 technical, batch PL14-0049 Purity: 96%	0, 500, 2000, and 8000 ppm (males) Equivalent to: 0, 29, 121 & 505 mg/kg bw/d 0, 500, 2000, and 5000 ppm (females) Equivalent to: 0, 37, 150 & 351 mg/kg bw/d 90-days continuous dosing Recovery period: 28-days (5/sex	**NOAEL** 500 ppm (29/37 mg/kg bw/day in M/F) Based on liver weight increases in females (16 % absolute & 17 % relative) and increased kidney weights in males (15 % absolute &14.5 %	One male (5000 ppm) was found dead on day 87 (undetermined cause); there were no clinical signs of toxicity at any dose **8000 / 5000 ppm (M/F)** 1 death (M) ↓ body weight: 9 %* (M) & 9.5 %** (F) ↓ body weight gain: 18 %** (M) & 23 %** (F) food efficiency in M: - 14 %** (main group) & + 22 % (recovery group) food efficiency in F: - 11 %* (main group) & + 55 % (recovery group) *Organ weights* ↑ liver weights in M: 21.5 %** (absolute) & 37 %**

Method, Species, test substance	Doses	NOAEL	Main adverse effects
Vehicle: acetone GLP OECD 408 (1998) & OECD 424 (1997) Deviations: None [redacted] (2016a) **Acceptable**	group)	relative) at 2000 ppm (Applicant also proposed a NOAEL of 2000 ppm)	(relative) ↑ liver weights in F: 22.5 %** (absolute) & 34 %** (relative) ↑ kidney weights in F: 17 %** (relative) *Histopathology - liver* Hepatocellular hypertrophy: 10/10 M (1 minimal, 6 mild, 3 moderate) and in 10/10 F (7 mild, 3 moderate) Macrovascular vacuolation 5/10 M (4 minimal, 1 moderate) Mild follicular cell hypertrophy: 3/10 (M) & 5/10 (F) *Histopathology - thyroid* Follicular cell hypertrophy (mild): 3/10 M & 5/10 F *Clinical chemistry* ↑ Cholesterol 40.5 %** (F) & 77 %** (M) ↑ globulin +11 %* and calcium +4.5 %* (F) **2000 ppm** *Organ weights* ↑ liver weights in females: 16 %* absolute & 17 %** relative ↑ kidney weights in males: 15 %* (absolute) & 14.5 %** (relative) *Histopathology - liver* Hepatocellular hypertrophy 1/10 F (mild) *Clinical chemistry* ↑ cholesterol +44 %**, globulin +11 % and calcium +4.5 %* (F) **500 ppm** No adverse effects **Recovery group (8000 / 5000 ppm)** ↑ food consumption 11 %** (M) & 4.5 % (F) ↑ relative liver weight 10 % (M) ↑ relative kidney weight 22 %** (M) Mild macro vascular vacuolation 1/5 (M) ↑ cholesterol 31 %** (F)

B.6.3.3.2. *Oral 90-day study in mice*

One study investigating the effect of 90-days' oral exposure of bixlozone in mice was available. Investigations to determine the plasma concentrations of bixlozone in mice were also included and are reported in more detail in Section B.6.1.1.3.

Study	A 90-Day Oral (Dietary) Toxicity and Plasma Concentration Measurement Study of F9600 Technical in CD-1 Mice
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016b)
Date performed	March - July 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105118
Guideline(s)	OECD 408(1998)
Deviations from the guideline	There were no deviations of significance
GLP	Yes (laboratory certified by National Authority)
Test material	F9600 Technical; batch PL14-0049 Purity 96%,
Method of analysis	*The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to the lack of explanation of the use of a non-linear calibration.* However, the method is considered sufficient for regulatory purposes.
Study acceptable	Yes

Material and Methods

In a GLP and OECD guideline study, doses of 0, 1000, 2250 & 5000 ppm (equivalent to 0, 180, 414 & 930 mg/kg bw/d and 0, 257, 583 &1185 mg/kg bw/d in males and females respectively) of bixlozone technical were administered *ad libitum* via the diet to Crl:CD1(ICR) mice for 90 days; the toxicology groups comprised 10 mice/sex and the toxicokinetic groups comprised 12 mice/sex.

For the toxicokinetic evaluation blood samples were collected from 3 animals/sex/group on study days 1, 21, 56, and 90. All animals were euthanized following blood collection. The liver from all toxicokinetic mice (excluding animals found dead) was examined macroscopically, weighed, and stored for possible future examination; the remainder of the carcass was discarded.

Results

Clinical signs and survival

There were no treatment-related deaths or clinical signs of toxicity. Two early deaths occurred during the toxicity study: one was due to head trauma (5000 ppm toxicity group, female) and the other was of undetermined cause (2250 ppm toxicity group, female). Three early deaths occurred during the toxicokinetic study: one was accidental (control toxicokinetic group, male) and the other two were of undetermined cause (5000 ppm toxicokinetic group, male; 1000 ppm toxicokinetic group, female). The lack of any dose-response suggests these deaths were unrelated to treatment.

Body weight changes and food consumption

There were no treatment-related effects on body weight gain or food consumption; the only statistically significant effect was a decrease in the overall body-weight gain of the 1000 ppm females (- 24 %). Owing to the absence of a clear dose-response HSE does not consider this to be a treatment-related effect. Minor sporadic alterations in the food consumption of treated groups compared with controls (in the first few weeks of the study only) were also not attributable to the test substance.

Overall there were no clear treatment-related effects on body weight development or food consumption after 90-days exposure in both sexes.

Haematological parameters, serum chemistry and urinalysis

With regard to haematological parameters, the only effect of note was a decrease in the group mean neutrophil count in females at 2250 ppm (- 42 %) and at 5000 ppm (- 40 %). In isolation, without effects on other WBC parameters, this decrease is considered unrelated to treatment.

With regard to serum chemistry, a statistically higher mean serum glucose (+ 19 %) and a lower mean phosphorous (- 17 %) were observed in females at top-dose; HSE considers these to be spurious findings not related to treatment with bixlozone as they did not show a dose-response relationship. All other changes in serum chemistry and haematology parameters were without statistical significance. Overall, there were no treatment-related and adverse changes in haematological, serum chemistry and urinalysis parameters.

Organ weight changes (

Table B 6.3.3.9*)*

The absolute and relative liver weights of mice in the treated groups were increased in comparison with those of the controls. In males there was a clear dose-related increase in liver weight, although statistical significance and adversity (> 15 %) were reached at top-dose only (+ 23 % absolute and + 23 % relative). A similar dose-related increase in liver weights was observed across all female dose groups, reaching statistical significance and adversity at 2250 ppm (+ 13 % absolute, + 17.5 % relative) and at 5000 ppm (+ 20 % absolute, + 21 % relative). HSE considered these increases to be treatment-related and adverse. The severity of the liver weight changes appeared to have increased slightly with the time of treatment in males (from 28- to 90-days' exposure), but they seemed in general similar for females.

Statistically significant reductions in the absolute weight of the epididymes were observed at 1000 ppm (- 10 %) and 5000 ppm (- 10 %): however since there is no clear dose-response and considering their magnitude and lack of histopathology, they are regarded as non-adverse.

There was a reduction in the absolute and relative weight of the adrenal gland in both sexes (to a lesser extent in males) at the top dose, which reached statistical significance in females only at 5000 ppm (+ 16 %). Toxicologically significant decreases (> 10%) in adrenal weight were also seen in mid-dose females. These decreases in adrenal weights were well within the laboratory HCD provided. Although these HCD (2009-2016) are not fully compliant with the data requirements laid out in Reg 283/2013 (section 5, point 3) because they cover 8 years, they are from the same laboratory and strain of mouse and from a reasonable number of studies performed around the date (2014) of the current study; thus, they are considered acceptable by HSE in a WoE approach. In addition, no associated histopathology was observed. Therefore, the adrenal weight changes are not considered treatment-related or adverse by HSE.

Overall treatment-related and adverse increases in liver weight were observed in females from 2250 ppm and in males at top-dose (5000 ppm).

Table B 6.3.3.9: Selected organ weights from the 90-day dietary study in mice

	Males					Females				
Dosage (ppm)	**0**	**1000**	**2250**	**5000**	**HCD Range (mean ± SD)**	**0**	**1000**	**2250**	**5000**	**HCD Range (mean ± SD)**
mg/kg bw/d	**0**	**180**	**414**	**930**		**0**	**257**	**583**	**1185**	
	Organ weights (g)									
Terminal body weight	37.9 ± 2.74	37.2 ± 2.04	37.9 ± 3.05	38.1 ± 2.57		29.3 ± 1.20	27.9 ± 2.46	28.2 ± 2.27	29.0 ± 1.59	
Liver	1.88 ± 0.26	1.92 ± 0.198	2.09 ± 0.29	**2.32** ± 0.16**		1.396 ± 0.08	1.43 ± 0.13	**1.575* ± 0.142**	**1.68** ± 0.185**	
% difference from control	-	1.9	11	**23**		-	2.3	**13**	**20**	
R		4.0	**11***	**23****		-	7.7	**17.5****	**21****	
Epididymides	0.114 ± 0.01	**0.103* ± 0.009**	0.108 ± 0.009	**0.1035* ± 0.0104**		-	-	-	-	
% difference from control	-	**-10**	-6.	**-10**		-	-	-	-	
R		-8	-6	**-10**						
Adrenal glands	0.0054 ± 0.00152	0.0052 ± 0.00148	0.0050 ± 0.00125	0.0048 ± 0.00061	0.0024 - 0.0091 (0.0048± 0.00139)	0.0107 ± 0.00149	0.0106 ± 0.00212	0.0094 ± 0.00116	**0.0090* ± 0.00106**	0.0053 - 0.0175 (0.0107 ±0.00229)
% difference from control	-	-4	-7	-11		-	-0.9	-12	**-16**	
R		0.0	-7	-7			3	-11	**-16**	

R % change in relative weight (organ weight to bodyweight) from control

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test

HCD date range for CD-1 mice – sub-chronic ([redacted] laboratory): Mar 2009 – May 2016; 11 studies/ 11 control groups for males; 10 studies/ 10 control groups for females

Microscopic changes (Table B 6.3.3.10)

The increases in liver weight observed above correlated to findings of minimal to mild hepatocellular hypertrophy seen in males from 2250 ppm and from 1000 ppm in females. At the top dose the findings were more prevalent in males with 10/10 being affected, whilst an incidence of 3/9 was observed in females. At 2250 ppm 4/10 males and 3/9 females presented early signs of hypertrophy (minimal to mild). The hypertrophy was characterised by enlarged individual hepatocytes with expanded eosinophilic cytoplasm, being most apparent in centrilobular and mid-zonal regions. There were no other microscopic findings.

Table B 6.3.3.10: Microscopic findings in the 90- day mouse study

	Microscopic examination							
	Males				Females			
Dosage (ppm)	**0**	**1000**	**2250**	**5000**	**0**	**1000**	**2250**	**5000**
mg/kg bw/d	**0**	**180**	**414**	**930**	**0**	**257**	**583**	**1185**
Liver (N)	10	10	10	10	10	10	9	9
Hepatocellular hypertrophy (N)	0	0	4	10	0	2	3	3
Minimal	0	0	3	1	0	2	3	1
Mild	0	0	1	9	0	0	0	2

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test

N number of animals examined

Overall hepatocellular hypertrophy was observed in males from the mid dose and in females from the lowest dose. The hypertrophy in females at the low dose and in males at the mid dose was not associated with adverse increases (> 15%) in liver weight or changes in clinical-chemistry parameters. Therefore, at these doses, the hypertrophy was not considered adverse.

Toxicokinetics (Table B 6.3.3.11)

Investigations to determine the plasma concentrations of bixlozone in mice were also included and are reported in more detail in Section B6.1.1.2. In short, systemic exposure to bixlozone in mice was observed, which increased with dose on study day 1 (with a slight decrease at 1000 ppm and 2250 ppm in females as an exception). Contrary to the effect observed in rats, exposure in mice was higher for males than for females by a magnitude of 2- to 9-fold; the magnitude of the sex difference was inversely proportional to the dose.

Exposure was generally lower on study days 21 and 56 (both sexes) and also on study day 90 (females). There was also no indication that bixlozone accumulates upon repeated dosing up to the end of the study.

Mean bixlozone technical in the plasma for the 5000 ppm dose group on study day 90 was determined as 45.4 ± 30.3 ng/mL and 21.1 ± 12.9 ng/mL for males and females respectively.

Table B 6.3.3.11: Plasma concentrations of bixlozone in the 90-day mouse study

	Males				Females			
Dose (ppm)	**0**	**1000**	**2250**	**5000**	**0**	**1000**	**2250**	**5000**
mg/kg bw/day	**0**	**180**	**414**	**930**	**0**	**257**	**583**	**1185**
	Mean plasma concentration ng/L							
Day 1		35.1 ± 10.4	139 ± 64.8	182 ± 51.7		48.5 ± 58.6	26.3 ± 11.9	140 ± 7.02
Day 21		48.3 ± 9.35	49.6 ± 12.2	98.6 ± 16.3		5.64 ± 0.09	18.8 ± 7.07	29.8 ± 5.87
Day 56		16.6 ± 3.49	46.8 ± 24.3	42.4 ± 14.1		8.92 ± 2.92	26.6 ± 18.5	36.6 ± 11.5
Day 90		16.5 ± 13.4	58.8 ± 39.5	45.4 ± 30.3		5.2 ± 0.3	13.9 ± 9.7	21.1 ± 12.9

Note: Blood samples were collected at approximately 06:00 hours at each interval. For values assigned as BLQ the most conservative value used was 5.00 ng/mL to estimate the mean plasma concentration unless all samples (n = 3) were assigned as BLQ.

Conclusion

Consistent with the effects observed in the 28-day mouse study and in the 28-day and 90-day rat studies, in this guideline 90-day study, the main effect of dietary administration of bixlozone in mice was an increase in liver weights associated with hepatocellular hypertrophy. HSE considers the liver weight increases from 2250 ppm in females (+ 17.5 % relative) and at the top dose of 5000 ppm in males (+ 20 % absolute & + 21 % relative) to be adverse in nature.

Based on these findings, a NOAEL of 1000 ppm for females (equivalent to 257 mg/kg bw/d) was identified from this study, with a LOAEL of 2250 ppm (583 mg/kg bw/d in females). The applicant proposed a NOAEL of 5000 ppm (the highest dose tested).

[redacted] (2016b)

Table B 6.3.3.12: Summary of the 90-day dietary study in the mouse

Method, Species, test substance	Doses	NOAEL	Main adverse effects
90 day, dietary Mouse, Crl:CD1(ICR), males & females, 10/sex/ toxicology group, 12/sex/toxicokinetic group F9600 technical, batch PL14-0049 Purity: 96% Vehicle: acetone GLP OECD 408 (1998) Deviations: none [redacted] (2016b) **Acceptable**	0, 1000, 2250, and 5000 ppm Equivalent to: Males: 0, 180, 414 &930 mg/kg bw/day Females: 0, 257, 583 & 1185 mg/kg bw/day	1000 ppm (257 mg/kg bw/d) Based on relative liver weight increases in females at 2250ppm (17.5 %) (The applicant proposed a NOAEL of 5000 ppm)	There were no test-substance related deaths or clinical signs of toxicity **5000 ppm** *Organ weights* ↑ absolute liver weights: 23 %** (M) & 20 %** (F) ↑ relative liver weights: 23 %** (M) & 21 %** (F) *Histopathology - liver* Hepatocellular hypertrophy in 10/10 M (1 minimal, 9 mild) Hepatocellular hypertrophy in 3/9 F (1 minimal, 2 mild) **2250 ppm** ↑ relative liver weights: 17.5 %** (F) ↑ absolute liver weights: 13 %* (F) Hepatocellular hypertrophy in 4/10 M (3 minimal, 1 mild) Hepatocellular hypertrophy in 3/9 F (minimal) **1000 ppm** No adverse effects observed

B.6.3.3.3. *Oral 90-day study in dogs*

One GLP and guideline study is available investigating the potential toxicity of bixlozone in Beagle dogs after 90-days repeated oral administration (capsule). In the 28-day dietary study the top dose of 30000 ppm led to severe palatability issues with the test substance, resulting in substantially lower body-weight gain; consequently a 7-day palatability study using encapsulated bixlozone was conducted with doses of 150, 350 and 550 mg/kg bw/day. The treatment was well tolerated up to the top dose. Therefore 30, 100, 300 and 750 mg/kg bw/day of bixlozone, administered in gelatine capsules containing neat test substance, were selected for males and females for this 90-day study.

Toxicokinetic evaluations to determine the plasma concentrations of bixlozone in dogs were included in the investigation and are reported in the ADME Section (please refer to Section B.6.1.1.3). Analyses were conducted using a validated high performance liquid chromatography method using UHPLC-MS/MS detection. The method validation is within a separate study (Lucarell, 2016c; WIL Research, USA; Study No. WIL-105142) and the data are presented in Volume 3, Section CA B.4, Point CA 4.1.2(c).

Study	A 90-Day Oral (Capsule) Dose Toxicity Study of F9600 Technical in Beagle Dogs
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016c)
Date performed	May 2015
Test facility	[redacted]
Report reference	Study no. [redacted]-105124
Guideline(s)	OECD Guideline 409 (1998)
Deviations from the guideline	None of significance
GLP	Yes
Test material	F9600 technical; batch PL14-0049 Purity 96%,
Method of analysis	*No method consideration is required for this study since bixlozone is administered orally using capsules.*
Study acceptable	Yes

Material and Methods

Bixlozone technical was administered in capsules to Beagle dogs (4 animals/sex/group) for 90 consecutive days. Doses of 30, 100, 300 and 750 mg/kg bw/day of bixlozone, administered in gelatine capsules containing neat test substance, were selected for males and females for the 90-day study. Tested doses were based on findings from the previous 28-day dietary study ([redacted], 2016a) and a subsequent 7-day oral (capsule) study ([redacted], 2015e). The control animals received the same number of empty gelatine capsules as the high-dose group.

Results

Clinical observations, survival, consumption, body weight

There were no treatment-related deaths or clinical signs of toxicity at any dose; neither were there any effects on body weight, body-weight gain or food consumption. Terminal body weights are shown in Table B 6.3.3.14.

Haematology, clinical chemistry and urinalysis (Table B 6.3.3.13)

Alterations in haematology parameters were noted in females at 750 mg/kg bw/day (top-dose), with white blood cell (WBC) count statistically significantly higher than controls (+ 37 % at week 6 and + 36 % at week 12/13). A statistically significant higher lymphocyte count in these females was also noted (+ 43 % at week 6 and + 39 % at week 12/13) along with a higher large unstained cell (LUC) count at week 6 (+ 150 %) compared to controls. There was a statistically significant lower red blood cell (RBC) count at 100 and 750 mg/kg bw/d on week 6 (- 10 % and - 5 % respectively). The effects on WBC, lymphocytes and LUC in top dose females are considered treatment-related and adverse, give the consistency across different parameters and time points. The effects on RBC are considered incidental given the lack of a dose-response and the small magnitude of the change.

With regard to clinical-chemistry parameters there were statistically significant lower alanine aminotransferase (ALT) values at week 6 in females from 300 mg/kg bw/day dose group; however, a decrease in this enzyme is not toxicologically significant and there was no clear dose-response. The only other observation with regard to clinical chemistry parameters was a higher globulin level and a resulting lower mean albumin/globulin ratio in the 30 mg/kg bw/day and the 750 mg/kg bw/day females; again there was no dose-response. Thus, these effects are considered unrelated to treatment.

All other haematology and clinical chemistry findings were sporadic findings that were not statistically significant, showed no clear dose-response. Urinalysis parameters were unaffected by treatment.

Overall there were some treatment-related and adverse effects in WBC, lymphocytes and LUC parameters seen in females only at the top-dose.

Table B 6.3.3.13: Selected haematology and clinical chemistry findings in the 90-day dog study

		Males					Females				
Dosage (mg/kg bw/day)		**0**	**30**	**100**	**300**	**750**	**0**	**30**	**100**	**300**	**750**
		Haematology and Coagulation									
WBC (thous/µL)	Week -1	9.84 ± 2.54	8.85 ± 0.96	9.18 ± 1.99	8.77 ± 3.17	10.10 ± 3.275	9.88 ± 1.31	8.24 ± 2.74	8.11 ± 1.62	8.02 ± 1.33	9.22 ± 1.07
	% from controls	-	-10	-7	-11	+3	-	-17	-18	-19	-7
	Week 6	7.82 ± 1.055	8.75 ± 1.47	8.43 ± 1.01	8.47 ± 0.57	9.05 ± 1.535	8.84 ± 0.67	7.72 ± 1.16	9.03 ± 0.89	9.05 ± 1.95	**12.1* ± 2.43**
	% from controls	-	+12	+8	+8	+16	-	-13	+2	+2	**+37**
	Week 12/13	8.86 ± 0.88	9.27 ± 1.08	8.56 ± 0.87	9.15 ± 0.59	10.19 ± 2.01	8.74 ± 0.94	10.01 ± 2.68	10.27 ± 3.19	9.01 ± 1.08	11.86 ± 2.01
	% from controls	-	+5	-3	+3	+15	-	+14	+17	+3	+36
RBC (mil/µL)	Week -1	6.56 ± 0.53	6.75 ± 0.72	7.00 ± 0.22	6.94 ± 0.57	6.67 ± 0.35	6.57 ± 0.009	6.72 ± 0.28	6.17 ± 0.42	6.81 ± 0.23	6.43 ± 0.39
	% from controls	-	+3	+7	+6	+2	-	+2	**-6**	+4	**-2**
	Week 6	6.60 ± 0.48	6.59 ± 0.55	7.00 ± 0.53	6.50 ± 0.59	6.06 ± 0.16	7.10 ± 0.37	6.62 ± 0.27	**6.41* ± 0.38**	6.80 ± 0.22	**6.30* ± 0.41**
	% from controls	-	-0.2	+6	-1.5	-8	-	-7	**-10**	-4	**-11**
	Week 12/13	6.68 ± 0.625	7.02 ± 0.41	7.24 ± 0.26	6.81 ± 0.19	6.72 ± 0.45	7.25 ± 0.30	6.87 ± 0.395	6.86 ± 0.13	6.66 ± 0.64	6.37 ± 0.445
	% from controls	-	+5	+8	+2	+0.6	-	-5	-5	-8	-12
Lymphocytes absolute (thous/µL)	Week -1	2.80 ± 0.55	2.77 ± 0.26	2.72 ± 0.21	3.05 ± 0.56	2.93 ± 0.24	2.64 ± 0.29	2.48 ± 0.89	2.72 ± 0.29	2.64 ± 0.41	3.20 ± 0.32
	% from controls	-	-1	-3	+9	+5	-	-6	+3	0	+21
	Week 6	2.68 ± 0.59	2.91 ± 0.93	2.65 ± 0.52	3.06 ± 0.498	2.65 ± 0.36	2.49 ± 0.37	2.18 ± 0.65	2.88 ± 0.27	2.58 ± 0.37	**3.55* ± 0.44**
	% from controls	-	+9	-1	+14	-1	-	+12	+15	+4	**+43**
	Week 12/13	2.76 ± 0.51	3.13 ± 0.85	2.82 ± 0.48	3.16 ± 0.295	3.09 ± 0.265	2.82 ± 0.62	2.66 ± 0.415	3.17 ± 0.18	2.80 ± 0.52	**3.92* ± 0.46**
	% from controls	-	+13	+2	+14	+12	-	+6	+12	-1	**+39**
LUC absolute (thous/µL)	Wk. 6	0.03 ± 0.010	0.04 ± 0.022	0.02 ± 0.005	0.03 ± 0.010	0.03 ± 0.005	0.02 ± 0.010	0.03 ± 0.005	0.03 ± 0.010	0.02 ± 0.015	0.05 ± 0.017
	% from controls										+150
		Serum Chemistry									
Albumin (g/dL)	Wk. 6	3.0 ± 0.08	2.9 ± 0.19	**3.3* ± 0.10**	3.1 ± 0.10	2.9 ± 0.10	3.2 ± 0.21	3.2 ± 0.08	3.2 ± 0.13	3.2 ± 0.19	3.0 ± 0.08
Globulin (g/dL)	Wk. 12/13	2.4 ± 0.21	2.2 ± 0.13	2.4 ± 0.23	2.3 ± 0.05	2.5 ± 0.10	2.1 ± 0.15	**2.4** ± 0.13**	2.2 ± 0.10	2.2 ± 0.05	**2.3* ± 0.10**
A/G RATIO	Wk. 12/13	1.30 ± 0.115	1.40 ± 0.082	1.43 ± 0.150	1.38 ± 0.050	1.28 ± 0.050	1.55 ± 0.058	**1.38* ± 0.096**	1.43 ± 0.096	1.48 ± 0.050	**1.30** ± 0.082**
ALT (U/L)	Wk. 6	30 ± 2.6	32 ± 17.9	25 ± 5.0	21 ± 4.1	21 ± 3.1	28 ± 3.9	25 ± 3.3	22 ± 2.4	**18** ± 2.6**	**20* ± 4.7**

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test

Thous Thousands

R % change in relative weigh (organ weight to bodyweight) from control

N Number of animals tested

BLQ Below the lower limit of quantitation

Ocular examination

Ocular examinations were carried out on the animals during acclimatisation and toward the end of the study period; there were no test substance related lesions and all findings were reported to be typical for laboratory dogs of this age and breed.

Organ changes – macroscopic examinations and weight changes (Table B 6.3.3.14)

There were no test substance-related macroscopic findings at the scheduled necropsy. Consistent with other repeated-dose toxicity studies conducted in the dog the main target organ of toxicity was the liver. Relative

and/or absolute liver weights were increased by > 15% in males from 300 mg/kg bw/day and in females from 100 mg/kg bw/day (+ 22, 21.5 and 46 % respectively). These increases are considered treatment-related and adverse.

There was an increase in uterus weights at 300 (+ 210 % relative) and 30 mg/kg bw/day (+ 312 % relative and + 337 % absolute); however there was no clear dose-response across the groups and no associated histopathology (see below), hence, the effect is not likely to be treatment-related.

A dose-related increase in the thyroid/parathyroid weights was observed in dogs after 90 days' exposure across all dose-groups in both sexes, which at 750 mg/kg bw/d was 43 % (relative) and 42.5 % (absolute) larger than controls in males and 25 % (relative) and 30 % (absolute) larger in females; however the results are variable since at no point statistical significance was reached. Histopathology examination did not correlate with the above findings, since only isolated cases of hyperplasia (C-cell, low grade) were noted in one male at 30 mg/kg bw/day (accompanied with mineralisation) and one female at 750 mg/kg bw/day. In the absence of histopathology, HSE considers them not adverse, with the possible exception of the increase seen at the top dose in females and from 300 mg/kg bw/day in males where the magnitude of the change justifies adversity.

A not statistically-significant but marked decrease in the relative weight of the prostate gland by 40% was noted at top-dose; the reduction in relative weight (> 10%) was also dose-dependent from 100 mg/kg bw/day. Histology of this organ revealed features consistent with immature prostate glands from 300 mg/kg bw/day (please refer to Table B.6.3.3.3.3.). Given the absence of effects on body weight development, the effects on the prostate weight (including immaturity) at least from 300 mg/kg bw/day (at which histopathology correlate was seen) are considered treatment-related and adverse.

It was lastly noted that the weight changes seen in the adrenal glands and thymus in the 28-day dietary study were not apparent in this 90-day study using capsules as the method of oral administration, which indicates that these organ changes were most likely the secondary consequence of the general toxicity (decreased body weights and body weight gains) caused by the unpalatability of the diet. There were no other statistically significant or biologically relevant effects on any other organ.

Overall, there were treatment-related and adverse effects on liver weight from 300 mg/kg bw/day in males and from 100 mg/kg bw/d in females, on prostate weight from 300 mg/kg bw/day in males and on thyroid weight from 300 mg/kg bw/day in males and at the top dose in females.

Table B 6.3.3.14: Selected organ weights from the 90-day dog study

	Males					Females				
Dosage (mg/kg bw/day)	**0**	**30**	**100**	**300**	**750**	**0**	**30**	**100**	**300**	**750**
	Organ weights (g)									
Terminal body weight (kg)	10.5 ± 0.76	10.7 ± 1.31	10.1 ± 1.37	11.2 ± 1.28	10.6 ± 1.27	7.3 ± 0.90	7.6 ± 0.83	7.6 ± 0.75	7.3 ± 0.89	7.7 ±0.52
Liver (g)	263 ± 20.6	265 ± 27.3	262 ± 31.3	306 ± 30.6	319 ± 37.8	180 ± 8.3	205 ± 16.8	**229* ± 20.0**	220 ± 33.5	**278** ± 34.0**
% difference from control	-	+1	-	+16	+21	-	+13	**+27****	**+22****	**+54****
R body weight	-	-0.6	+3	+10	**+20****	-	+8	**+22****	**+21.5****	**+46****
Thyroid/Parathyroid	0.712 ± 0.100	0.867 ± 0.111	0.858 ± 0.268	1.001 ± 0.148	1.012 ± 0.225	0.593 ± 0.179	0.576 ± 0.123	0.655 ± 0.157	0.650 ± 0.118	0.773 ± 0.346
% difference from control	-	+22	+20	+40	+43	-	-3	+10	+10	+30
R body weight	-	+14	+14	+29	+43	-	0	+12.5	+12.5	+25
Uterus/Cervix			- -			3.56 ± 0.54	**15.56** ± 5.93**	3.71 ± 0.78	10.96 ± 4.54	6.00 ± 4.25
% difference from control			-			-	**+337**	+4	**+208**	**-+68**
R body weight			- -			-	**+312****	0	**210***	54
Prostate	7.09 ± 2.34	9.15 ± 2.73	5.99 ± 1.11	5.74 ± 2.21	4.18 ± 1.64					
% difference from control	-	+29	-15.5	-19	-41					
R body weight	-	+36.5	-12	-26.5	-43					

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test
R % change in relative weigh from control

Microscopic findings (Table B 6.3.3.15)

There were no treatment-related macroscopic findings.

Hepatocellular hypertrophy was present in 2/4 males at the top dose (to a minimal extent), characterised by an enlargement of the hepatocytes in the centrilobular region with elevated amounts of eosinophilic cytoplasm. The hepatocellular hypertrophy in the top-dose males correlates with the increases in liver weight noted at this dose; however there were no findings of hypertrophy in the top dose females where the greatest increases in liver weights were observed.

Immature development of the prostate was observed from 300 mg/kg bw/day, affecting up to 3 (out of 4) animals at the top-dose; the finding correlated well with the dose-dependent reduction in the relative prostate weight observed for these animals.

Overall there was minimal hepatocellular hypertrophy observed in males only at the top dose of 750 mg/kg bw/day; the findings are not as severe as those observed in the 28-day oral toxicity study, which could be partly explained by the administration method used in this study (oral capsules vs diet in the 28-day study). There was also immature development of the prostate from 300 mg/kg bw/day.

Table B 6.3.3.15: Microscopic findings in the 90-day dog study

	Males					Females				
Dosage (mg/kg bw/day)	**0**	**30**	**100**	**300**	**750**	**0**	**30**	**100**	**300**	**750**
	Microscopic Examination									
Liver (N)	4	4	4	4	4	4	4	4	4	4
Hypertrophy, hepatocellular, centrilobular	0	0	0	0	2	0	0	0	0	0
Minimal	-	-	-	-	2	-	-	-	-	-
Prostate (N)	4	4	4	4	4					
Immature development	0	0	0	1	3					

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test
N Number of animals tested

Toxicokinetics (Table B 6.3.3.16)

Systemic exposure was noted in all dogs (with some exceptions) at 300 and 750 mg/kg bw/day and in at least 2/4 dogs at 30 and 100 mg/kg bw/day. Plasma concentrations of bixlozone increased in line with dose at the 4-hour measurement although intra-group variability was high. For example, the 7.5-fold dose increase from 100 to 750 mg/kg bw/day, resulted in a corresponding 4.3- to 110-fold increase in plasma concentration of bixlozone on all days. There was no apparent difference between males and females (aside from on study day 30 when the plasma concentrations were below the limit of quantification); the concentrations observed do not indicate that there is any accumulation of bixlozone in plasma after repeated-exposure in dogs.

Table B 6.3.3.16: Plasma concentrations of bixlozone in the 90-day dog study

	Toxicokinetics									
	Males					Females				
Dosage (mg/kg bw/day)	**0**	**30**	**100**	**300**	**750**	**0**	**30**	**100**	**300**	**750**
	Mean concentration in plasma (ng/mL)									
Day 0		7.7 ± 2.9	8.0 ± 4.0	54.6	611		BLQ	8.5 ± 4.0	26.3	355
Day 38		11.0 ± 9.9	BLQ	21.1	71.9		BLQ	12.5 ± 4.0	10.9	54.4
Day 89		BLQ	11.0 ± 9.9	15.4	42.6		BLQ	8.5 ± 4.4	15.6	32.3

BLQ Below the lower limit of quantitation. For values assigned as BLQ the most conservative value used was 5.00 ng/mL to estimate the mean plasma concentration unless all samples (n = 3) were assigned as BLQ

Conclusion

In a guideline study, the main effect seen after administration of bixlozone technical (capsule) for 90-days in dogs was in the liver, with treatment-related and adverse effects observed from 300 mg/kg bw/day in males and

100 mg/kg bw/day in females. Liver weight increases were most extensive in females at top-dose (+ 54 % absolute and + 46 % relative, both statistically significant) compared to males (+ 20 % absolute and + 21 % relative, the last being statistically significant); however only males showed an associated minimal hepatocellular hypertrophy at top-dose (2/4 males).

Although there were no clear changes in clinical chemistry or histopathological findings that would indicate hepatotoxicity in this study, HSE considers that the magnitude of the increases in liver weights (particularly in females) is treatment-related and adverse. There were also adverse reductions in prostate weight with associated immaturity from 300 mg/kg bw/day and adverse increases in thyroid weight at the top dose of 750 mg/kg bw/day in females and from 300 mg/kg bw/day in males and effects on WBC, lymphocytes and LUC parameters in top dose females.

Overall a NOAEL of 30 mg/kg bw/day has been set from this study based on a statistically significant increase in relative liver weights > 15 % observed at the LOAEL of 100 mg/kg bw/day in females; the applicant considered the liver findings to be adaptive and non-adverse and proposed to set the NOAEL at the highest dose tested of 750 mg/kg bw/day.

[redacted] (2016c)

Table B 6.3.3.17: Summary of the 90-day oral (capsule) study in the dog

Method, Species, test substance	Doses	NOAEL	Main adverse effects
90 day, capsule Dogs, Beagle, males & females, 4/sex/group F9600 technical, batch PL14-0049 Purity: 96% Vehicle: none GLP OECD 409 (1998) [redacted] (2016c) **Acceptable**	0, 30, 100, 300, and 750 mg/kg/day	30 mg/kg bw/day for females Based on liver weight increases in females at 100 mg/kg bw/d (27 % absolute & 22 % relative) (The applicant proposed a NOAEL of 750 mg/kg bw/day)	There were no treatment-related deaths or clinical signs of toxicity; there was no effect on body weight or food consumption at any dose levels. **<u>750 mg/kg bw/day</u>** *Organ weights* ↑ absolute liver weights: 54 %** (F) & 21 % (M) ↑ relative liver weights: 46 %** (F) & 20 %** (M) ↑ relative thyroid weight: 54 % (F) & 21 % (M) ↓ prostate weight: absolute 41 % and relative 43 % and associated immaturity *Histopathology - liver* Hepatocellular hypertrophy in 2/4 males (minimal) *Clinical chemistry* ↑ WBC (37 %* wk. 6), ↑ lymphocytes (43 %* wk. 6 & 39 %* wk. 12/13), ↑ LUC (+150 % wk 6) in F **<u>300mg/kg bw/day</u>** ↑ relative liver weight: 21.5 %** (F) ↓ abs (19%) and rel (26.5 %) prostate weight and associated immaturity **<u>100 mg/kg bw/day</u>** ↑ liver weights in F (27 %* absolute, 22 %** relative) **<u>30 mg/kg bw/day</u>** No adverse effects observed.

B.6.3.4. Oral 12-month study in dogs

One GLP and OECD guideline study is available investigating the potential toxicity of bixlozone in Beagle dogs, after 12 months' repeated oral administration (capsule).

Study	A 12-Month Oral (Capsule) Dose Toxicity Study of F9600 Technical in Beagle Dogs
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2017)
Date performed	March 2016- March 2017
Test facility	[redacted] (formerly [redacted] facility)
Report reference	Study no. [redacted]-105125
Guideline(s)	OECD Guideline 452 adopted September 2009
Deviations from the guideline	None of significance
GLP	Yes
Test material	F9600 technical; batch PL14-0049 Purity 96%,
Method of analysis	No method consideration is required for this study since bixlozone is administered orally using capsules.
Study acceptable	Yes The OECD Guideline 452 has been reviewed since the conduction of the study (latest adoption: 25th June 2018). This guideline is intended primarily to studies carried out in rodent species. Should such studies be required in non-rodent species, the principles and procedures outlined in this Guideline, together with those outlined in OECD TG 409, Repeated Dose 90 day Oral Toxicity Study in Non-Rodents may also be applied, with appropriate modifications, as outlined in the OECD Guidance Document No. 116 on the Design and Conduct of Chronic Toxicity and Carcinogenicity Studies (ENV/JM/MONO(2011)47). The latter document further specifies that tests of longer duration than 3 months using typical non-rodent species such as the dog do not have a substantial added value for making regulatory decisions. As such a 12-months study is no longer part of the Commission Regulation (EU) No 283/2013 data requirements for toxicological and metabolism studies. However, in this instance, HSE considers the study acceptable and to be relied upon since some of the findings observed in this study have been used in the weight of evidence assessment of the toxicological profile of bixlozone upon repeated dosing.

Material and Methods

Bixlozone technical was administered in capsules to Beagle dogs (4 animals/sex/group) for 12 consecutive months. Tested doses were 20, 100 and 500 mg/kg bw/day of bixlozone for males and females. The control animals received the same number of empty gelatine capsules as the high-dose group.

Results

Clinical observations, survival, body weight, food consumption

All animals survived to the scheduled necropsy. There were no biologically relevant test substance-related effects on clinical observations, body weights and food consumption.

Haematology (Table B 6.3.4.1)

Alterations in haematology parameters were noted in males at top-dose (500 mg/kg bw/day): at week 26 the white blood cell (WBC) count was statistically significantly higher than controls, which was attributable to higher mean absolute monocyte (statistically significant) and lymphocyte (not statistically significant) counts; at week 52 the WBC count remained high (albeit not statistically significant) with an increased lymphocyte count reaching statistically significance compared to controls.

Coagulation parameters were also affected with a dose-response increase in Prothrombin time (PT) noted in males at week 52, reaching statistically significance at 500 mg/kg bw/day group males. At week 26, increase in PT was also statistically significant at top-dose.

Overall WBC and coagulation parameters were adversely affected by treatment at the top-dose (500 mg/kg bw/day) in males at week 26 and 52 of the study. These effects appear to be more pronounced than those noted in the 90-day dog study ([redacted] (2016c)).

Clinical chemistry, urinalysis (Table B 6.3.4.1)

Several clinical chemistry parameters changes were observed at week 26 and 52. Glucose values were statistically significant lower for all treated males compared to controls at week 52; similar results were seen at week 26 but to a lesser extent; the reductions in glucose levels did not show a clear dose-response and such effect was not seen in females. There were also 2 mild cases of inflammation of the Langheran islets of the pancreas (which regulate glucose levels through hormone production) noted in the top dose males. However, HSE notes that there was no clear correlation between the glucose levels and the incidence of pancreatic inflammation since the reductions in glucose levels were similar across all tested groups whilst pancreatic inflammation was observed only at the top dose. Thus, in the absence of a dose-response and considering that similar effects were not seen in the previous 28-day and 90-day dog studies up to higher doses (Sections B.6.3.2.3 & B.6.3.3.3), it is most likely this decrease in glucose levels in males is unrelated to treatment.

A statistically significant lower mean albumin value was noted at 500 mg/kg bw/day in males at week 52 whilst statistically significant lower mean A/G ratio values were noted at 20 and 500 mg/kg bw/day in females; the relevance of such changes remains uncertain, since there is no evident dose-response.

Ophthalmic findings, macroscopic observations, organ weights, microscopic findings (Table B 6.3.4.1)

There were no test substance-related ophthalmic or macroscopic findings.

With regard to organ weights, the only observation to be noted was a statistically significant decrease in absolute spleen weight in females only at top-dose. However, the relative spleen weight values for the treated females did not show any clear dose-response and there were no associated microscopic findings; thus, it is not considered treatment-related. It is noted that there were no increases in liver weight and/or hypertrophy up to 500 mg/kg bw/day in this 1-year study. Thus, in contrast with the 90-day study, there were no notable effects seen in the liver or prostate.

With regard to microscopic findings the only observation to be noted was an increased incidence of mild inflammation of the pancreas in males at the top-dose of 500 mg/kg bw/day only. The finding was characterised by focal mononuclear cell infiltrates with fibrosis and scant necrotic debris involving Langheran's islet and adjacent acini. The study report considered the finding to be unrelated to treatment due to its focal distribution and from documented published literature describing it as a spontaneous change in dogs (Greaves, P. 2012). HSE notes that the microscopic pancreatic findings seen at the top-dose in males did not correlate with reductions in glucose levels that could be attributed to treatment. In addition, although no sex difference was seen in blood bixlozone levels, there were no pancreatic findings in females up to the top-dose. Thus, the WoE assessment shows that overall the mild chronic focal inflammation of the pancreas observed in males at the top dose is unlikely to be related to treatment and adverse.

Overall there were no toxicologically significant microscopic findings noted in this study.

Table B 6.3.4.1: Selected Findings in the 12 month dog capsule study with bixlozone technical

		Males				Females			
Dose (mg/kg bw/day)		**0**	**20**	**100**	**500**	**0**	**20**	**100**	**500**
Haematology and Coagulation									
WBC ($\times 10^3/\mu L$)	**Week -1**	8.46 ± 2.128	8.05 ± 1.709	10.19 ± 1.323	8.95 ± 1.001	7.17 ± 0.780	8.92 ± 2.762	9.36 ± 2.560	7.87 ± 1.169
	Week 26	8.33 ± 0.529	8.56 ± 1.675	9.68 ± 0.856	**11.23 ± 1.801***	8.79 ± 2.006	7.99 ± 0.386	9.94 ± 3.754	10.38 ± 3.334
	Week 52	7.39 ± 0.982	9.12 ± 2.555	7.81 ± 0.731	9.37 ± 0.924	7.99 ± 2.182	9.39 ± 3.713	7.29 ± 1.803	8.29 ± 1.336
PT (seconds)	**Week -1**	7.7 ± 0.21	7.6 ± 0.28	7.4 ± 0.13	7.7 ± 0.33	7.3 ± 0.29	7.5 ± 0.10	7.7 ± 0.56	7.6 ± 0.61
	Week 26	7.4 ± 0.26	7.5 ± 0.28	7.4 ± 0.22	**8.0 ± 0.21***	7.4 ± 0.1	7.7 ± 0.10	7.7 ± 0.88	7.7 ± 0.53
	Week 52	7.7 ± 0.25	8.1 ± 0.42	8.2 ± 0.34	**8.7 ± 0.29****	7.8 ± 0.29	8.0 ± 0.33	8.3 ± 0.56	8.1 ± 0.50
Lymphocytes absolute ($\times 10^3/\mu L$)	**Week -1**	2.65 ± 0.324	2.67 ± 0.881	3.08 ± 0.340	3.15 ± 0.641	2.45 ± 0.552	2.56 ± 0.441	2.58 ± 0.933	2.93 ±0.374
	Week 26	2.59 ± 0.235	2.59 ± 0.573	3.10 ± 0.532	3.48 ± 1.015	2.33 ± 0.176	2.43 ± 0.426	2.46 ± 0.795	3.02 ± 0.663
	Week 52	2.19 ± 0.421	2.22 ± 0.188	2.56 ± 0.559	**3.40 ± 0.383 ****	2.11 ± 0.280	2.04 ± 0.341	2.32 ± 0.522	2.76 ± 0.628
Monocytes absolute ($\times 10^3/\mu L$)	**Week -1**	0.71 ±0.365	0.56 ± 0.163	0.80 ± 0.101	0.59 ± 0.110	0.44 ± 0.141	0.55 ±0.303	0.70 ± 0.241	0.39 ± 0.113
	Week 26	0.41 ± 0.067	0.45 ± 0.148	0.48 ± 0.114	**0.69 ± 0.120***	0.42 ± 0.143	0.33 ± 0.095	0.42 ± 0.188	0.46 ± 0.202
	Week 52	0.40 ± 0.150	0.49 ± 0.132	0.38 ± 0.130	0.46 ± 0.110	0.31 ± 0.095	0.51 ± 0.407	0.28 ± 0.047	0.34 ± 0.079
Serum Chemistry									
Albumin (g/dL)	**Week -1**	2.7 ± 0.21	2.8 ± 0.13	2.8 ± 0.08	2.6 ± 0.33	2.9 ± 0.08	2.9 ± 0.12	2.8 ± 0.16	2.9 ± 0.13
	Week 26	3.2 ± 0.10	3.1 ± 0.08	3.1 ± 0.10	2.9 ± 0.15	3.1 ± 0.10	3.0 ± 0.06	3.0 ± 0.17	3.0 ± 0.10
	Week 52	3.4 ± 0.15	3.2 ± 0.10	3.3 ± 0.06	3.1 ± 0.12*	3.3 ± 0.17	3.2 ± 0.10	3.3 ± 0.13	3.1 ± 0.14
A/G ratio	**Week -1**	1.20 ± 0.082	1.15 ± 0.129	1.25 ± 0.129	1.18 ± 0.171	1.33 ± 0.096	1.30 ± 0.141	1.23 ± 0.126	1.28 ± 0.150
	Week 26	1.13 ± 0.050	1.18 ± 0.096	1.20 ± 0.141	1.15 ± 0.129	1.28 ± 0.096	1.23 ± 0.050	1.23 ± 0.126	1.18 ± 0.189
	Week 52	1.25 ± 0.058	1.20 ± 0.082	1.33 ± 0.150	1.20 ± 0.082	1.48 ± 0.126	1.28 ± 0.050*	1.35 ± 0.129	1.23 ± 0.096*
Glucose (g/dL)	**Week -1**	107 ± 4.6	100 ± 4.1	105 ± 5.4	104 ± 5.7	96 ± 5.4	99 ± 7.7	99 ± 8.2	100 ± 8.1
	Week 26	106 ± 2.2	**94 ± 5.6***	100 ± 3.8	**96 ± 8.1***	96 ±4.1	90 ± 9.7	102 ± 2.6	98 ± 6.8
	Week 52	101 ± 2.6	**93 ± 2.2****	**94 ± 2.2****	**95 ± 1.9****	93 ± 6.2	93 ± 5.2	93 ±7.9	96 ± 5.0
Organ weights									
Final body weight (kg)		11.5 ± 2.77	10.1 ± 1.17	10.1 ± 0.54	10.2 ± 0.64	8.5 ± 1.27	8.7 ± 1.43	9.1 ± 0.94	7.7 ± 1.10
Liver weight (g)		244 ± 56	219 ± 23	226 ± 4.3	243 ± 34	195 ± 21	196 ± 23	215 ± 13	198 ± 43*
% from controls		-	-10	-7	-0.4		+0.8	+10	+2
Liver weight/final body weight (g/100 g)		2.13 ± 0.20	2.17 ± 0.06	2.23 ± 0.09	2.37 ± 0.20	2.31 ± 0.14	2.28 ± 0.37	2.39 ± 0.33	2.55 ± 0.22
% from controls		-	+1.6	+4.4	+10.8	-	-1.1	+3.2	+10
Spleen weight (g)		190.5 ± 35	184.4 ± 38	147 ± 49	167.5 ± 13	142 ± 27	130 ± 13	155 ± 23	**96.7 ± 28***
Spleen weight/final body weight (g/100 g)		1.76 ± 0.55	1.84 ± 0.4	1.46 ± 0.5	1.64 ± 0.2	1.67 ± 0.2	1.53 ± 0.3	1.71 ± 0.2	1.25 ± 0.3
% from control		-	+5	-17	-6	-	-9	+2	-25

	Males				Females			
Dose (mg/kg bw/day)	**0**	**20**	**100**	**500**	**0**	**20**	**100**	**500**
Microscopic examination								
Pancreas N	4	4	4	4	4	4	4	4
Inflammation, chronic, focal	0	0	0	2	0	0	0	0
Mild	-	-	-	2	-	-	-	-

* Significantly different from the control group at 0.05 using Dunnett's test
** Significantly different from the control group at 0.01 using Dunnett's test
WBC White blood cells
PT ratio Prothrombin time
A/G Albumin to globulin
G/dL Grams/decilitre
Meq/L Milliequivalents per litre

Conclusion

In a GLP and OECD guideline study, the main treatment-related effect seen after administration of bixlozone technical (capsule) for 12 months in dogs was a change in haematology parameters in males only, where WBC and PT were affected at the top-dose (500 mg/kg bw/day) at week 26 and 52 of the study; similar but less extensive findings were observed after 90-day exposure ([redacted] (2016c)). Contrasting with the previous studies conducted with dogs, there were no notable liver or prostate effects up to 500 mg/kg bw/day.

Based on these findings, a NOAEL of 100 mg/kg bw/day has been identified from this study. The applicant proposed a NOAEL of 500 mg/kg bw/day.

[redacted] (2017)

Table B 6.3.4.2: Summary of the 12 months dietary study in the dog

Method, Species, test substance	Doses	NOAEL	Main adverse effects
12 months, capsule Dogs, Beagle, males & females, 4/sex/group F9600 technical, batch PL14-0049 Purity: 96% Vehicle: none GLP OECD 409 (1998) Deviations: none [redacted] (2017) **Acceptable**	0, 20, 100, and 500 mg/kg/day	100 mg/kg bw/day for females Based on haematological changes in males at 500 mg/kg bw/day (WBC, PT and lymphocytes absolute) (The applicant proposed a NOAEL of 500 mg/kg bw/day)	There were no treatment-related deaths or clinical signs of toxicity; there was no effect on body weight or food consumption at any dose levels. **500 mg/kg bw/day** ↑ WBC (+35 % week 26*; +27 % week 52) ↑ monocyte absolute (+55% week 26*;+15 % week 52) ↑ lymphocytes absolute (+34 % week 26; +55 % week 52**) ↑ PT (+8 % week 26*; +13 % week 52**) in males **100 & 20 mg/kg bw/day** No adverse effects observed.

B.6.3.5. Other routes

A GLP and OECD guideline dermal 21-day repeated-dose toxicity study in the rat is available.

Study	A 21-Day study of F9600 by Dermal Application in Sprague-Dawley Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016)
Date performed	2016
Test facility	[redacted]
Report reference	Study no. [redacted]-105181
Guideline(s)	OECD 410 (1981)
Deviations from the guideline	None
GLP	Yes (lab certified by National Authority)
Test material	F9600 Technical Batch PL14-0049; Purity 96.0%
Method of analysis	No method consideration is required for this study since bixlozone is administered via the dermal route.
Study acceptable	Yes

Material and Methods

Bixlozone technical was administered dermally to Crl:CD(SD) rats (10 animals/sex/group) for 21 consecutive days (6-7 hours of exposure/day). The test substance was moistened with deionised water and applied directly to the skin once daily at doses of 100, 300 and 1000 mg/kg bw/day respectively. The control animals received the same amount of deionised water used to moisten the test substance for animals in the high dose group on a comparable regimen. A method of analysis for the test substance concentrations is not required for this study.

Results

Clinical and dermal observations, ocular examination and survival

One control female was found dead on study day 3 at the time of the 6-7 hour post-dosing unwrap. The death was attributed to the female being wrapped up too tightly. The animal was replaced with another acclimation female which was administered the vehicle from study day 4. All remaining animals survived to scheduled necropsy.

There were no test substance-related clinical or dermal observations. At various intervals observations related to animals being wrapped too tightly were noted, but these are not considered treatment-related. No ophthalmic lesions indicative of toxicity were observed in any of the test substance-treated groups compared to control groups.

Bodyweight, bodyweight gain and food consumption ***(Error! Reference source not found.)***

There were no test substance or dose-response related effects observed on mean body weight , body weight gains and food consumption in treated groups compared to the control groups.

Clinical pathology - Haematology and coagulation, serum chemistry and urinalysis ***(Error! Reference source not found.)***

There were no test substance or dose-response related alterations in haematology, coagulation and serum chemistry parameters in treated groups compared to the control groups. There was a statistically significant decrease in cholesterol in females at the mid dose. However, given the lack of a dose response, this change was considered an incidental finding. It is also noted that in the oral studies, cholesterol levels were increased rather than decreased and were affected in both sexes.

With regard to urinalysis parameters, the only notable change seen was for the specific gravity value, which was statistically significantly lower, whilst the total urine volume was higher at 1000 mg/kg bw/day in females only. Since there is no other relevant test substance-related change observed in serum chemistry, gross or histological observations suggestive of nephrotoxicity, the changes are not considered to be adverse.

Anatomic pathology – organ weights

There were no statistically different, test substance or dose-response related alterations in organ weights in the treated groups compared to the control groups, including in the target organs identified after repeated oral administration of bixlozone (liver, kidney).

Anatomic pathology – macroscopic and microscopic examinations ***(Error! Reference source not found.)***

There was an increased incidence of liver necrosis across all groups including controls, which was considered secondary to wrapping of the torso given the absence of treatment-related changes in liver weights or serum chemistry. Moreover similar liver changes associated with wrapping of the torso have been described in the literature (Parker, G.A.; Gibson W. B. Liver lesions in rats associated in wrapping of the torso. *Toxicologic Pathology* **1995,** *23*, pp 507-512).

Overall there were no test substance-related histological changes observed in the liver.

Table B 6.3.5.1: Selected Findings in the 21 day rat dermal study with bixlozone

	Males				Females			
Dose (mg/kg bw/day)	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
	Cumulative body weight change							
Day -1 to 6	35 ± 7.1	28 ± 13.6	34 ± 13.1	31 ± 12.0	17 ± 9.1	17 ± 7.3	16 ± 6.8	17 ± 5.5
Day -1 to 13	73 ± 12.1	63 ± 19.1	66 ± 22.6	63 ± 19.2	31 ± 10.8	30 ± 10.9	30 ± 8.9	32 ± 5.8
Day -1 to 20	100 ± 19.2	90 ± 24.4	82 ± 31.7	85 ± 25.6	41 ± 11.5	37 ± 10.1	45 ± 13.4	39 ± 7.8
% from controls (day 1-20)	-							
	Serum Chemistry							
Cholesterol (mg/dL)	54 ± 9.1	53 ± 7.9	54 ± 14.8	52 ± 11.5	62 ± 10.2	65 ± 12.2	48 ± 10.4*	55 ± 12.4
% from control		-1.9	0.0	-3.7		4.8	-22.6	-11.3
	Urinalysis							
Specific gravity	1.03 ± 0.0155	1.031 ± 0.0122	1.03 ± 0.0123	1.028 ± 0.0103	1.048 ± 0.0194	1.041 ± 0.0183	1.034 ± 0.0112	1.022 ± 0.0157**
pH	6.4 ± 0.47	6.5 ± 0.28	6.3 ± 0.26	6.2 ± 0.24	6.0 ± 0.41	6.1 ± 0.28	6.1 ± 0.28	6.1 ± 0.16
Total volume (mL)	11.9 ± 6.69	9.9 ± 6.35	8.6 ± 3.37	7.8 ± 3.99	5.3 ± 5.08	5.5 ± 2.8	7.0 ± 2.87	13.5 ± 7.89**
Microscopic examination – liver necrosis								
Total number examined	10	4	1	10	10	3	5	10
Number affected	3	3	1	3	3	2	2	3
Minimal	1	NA	1	0	1	1	NA	2
Mild	None	2	NA	1	1	NA	2	1
Moderate	1	1	NA	2 (focal)	1	1	NA	None
Marked	1	NA	NA	None				

* = Significantly different from the control group at 0.05 using Dunnett's test
** = Significantly different from the control group at 0.01 using Dunnett's test
N/A = not applicable.

Conclusion

In this GLP and OECD guideline study the dermal administration of bixlozone *via* direct topical application to the skin of Crl:CD(SD) rats at dose levels of 100, 300 and 1000 mg/kg bw/day for 21 days was well tolerated at all doses. There was no test-substance related effects on clinical observations, dermal observations, body weights, food consumption, clinical pathology parameters or organ weight changes including in the liver and kidney identified as target organs following oral administration. There were no test substance-related ophthalmic, macroscopic or microscopic findings.

Based on the absence of toxicity at any dose level, 1000 mg/kg bw/day (the highest dose level tested) was considered the NOAEL.

[redacted] (2016)

Table B.6.3.5.2 Summary of the 21-day dermal study in the rat

Method, Species, test substance	Doses	NOAEL	Main adverse effects
21 day, dermal Rat, Crl :CD9(SD), males & females, 10/sex/toxicology group (Inc. control), GLP OECD 410 (1981) Deviations : None Bixlozone technical, batch PL14-0049 Purity: 99.2% [redacted] (2016) **Acceptable**	0, 100, 300, and 1000 mg/kg bw/day	1000 mg/kg bw/day based on no adverse effects observed at the highest dose tested (The applicant proposed a NOAEL of 1000 mg/kg bw/day)	There were no deaths or clinical signs of toxicity **1000, 300 and 100 mg/kg bw/day** No adverse effects observed.

B.6.3.6. Summary of short-term toxicity

The short-term oral toxicity of bixlozone has been extensively investigated in GLP and OECD guideline compliant repeated-dose toxicity studies in rats, mice and dogs following 28- and 90-days' dietary exposure; a 12-month oral (capsule) study conducted in dogs is also available. This study, which is no longer required in Reg 283/2013, is considered acceptable and is relied upon as it has been used by HSE for a WoE assessment of the short-term toxicity of bixlozone. Moreover, 7-day palatability studies have been conducted in rats, mice and dogs; these studies are not GLP or OECD compliant, however they are reported in this Section as supplementary information. Considering other routes of exposure, a 21-day dermal study in rats is available for bixlozone. Further information on the short-term oral toxicity of bixlozone can also be extracted from the 2-generation reproductive toxicity study (see Section B.6.6.) and from the long-term toxicity studies (see Section **Error! Reference source not found.**.) conducted in rats and mice and have been taken into consideration in this summary.

The liver has been identified as a clear target organ in all species investigated: there were increases in relative and absolute liver weights accompanied in some instances with minimal to moderate hepatocellular hypertrophy. The toxicological significance of the effects on the liver has been assessed by HSE using a weight-of-evidence approach (WoE), with a clear distinction being made between effects that are clearly adverse and those which are potentially adaptive. This assessment has been carried out in line with the Technical Agreements for Biocides (TAB) entry, agreed at the Biocide Working Group-IV-2018 meeting (WGIV2018_TOX_6-2); this paper describes a WoE approach for the evaluation of liver effects in repeated-dose toxicity studies based on several international reviews on liver effects (JMPR 2006 and 2015). Hepatocellular hypertrophy is typically related to increased functional capacity of the liver which allows the maintenance of homeostasis in the organism after xenobiotic exposure. A general increase in the size of the liver is observed (owing to cell enlargement and fluid accumulation); this is considered a potentially beneficial, adaptive response. However, there is the potential that the capacity of the homeostatic mechanisms may be exceeded and in these cases the organism would be unable to return to its previous state once exposure has ended (thus constituting an adverse response). Hypertrophy as an adaptive response should not be accompanied by adverse histopathology (necrosis, apoptosis, pigment deposition or hyperplasia), or by substantial changes in clinical chemistry indicative of liver toxicity (decreased albumin or increased activities of ALT, AST, ALP, GGT, bilirubin or cholesterol). In line with the TAB entry, relative liver weight increases up to 15 %, that are not accompanied by other signs of liver dysfunction, have been considered by HSE to be an adaptive rather than an adverse response in the evaluation of the liver effects of bixlozone (see table below).

By means of these criteria it can be seen that the effects exerted upon the liver by bixlozone become adverse in the rat at 150 mg/kg bw/day (females, 90-days' exposure), at 583 mg/kg bw/day in the mouse (females, 90-days' exposure) and at 100 mg/kg bw/day in the dog (females, 90-days' exposure).

It would appear that the rat and the dog are more sensitive than the mouse to the liver effects, and that the female is the most sensitive sex across all species. These findings are concordant with the toxicokinetic evaluations which showed that systemic exposure was higher in female rats compared to male rats (the top dose was indeed set lower for females in the 90-day rat study) but was greater in male mice compared to female mice (please refer to Section B.6.1.1.3 for more details). Furthermore, the effect on liver weights and histopathological incidence and severity did not appear to increase to any great extent with the duration of treatment in any species, which is supported by toxicokinetics evidence indicating that bixlozone and its metabolites did not accumulate in plasma or tissues following 14 days repeated dosing (Section **Error! Reference source not found.** Summary of ADME studies).

Table B 6.3.6.1: Summary of the liver effects of bixlozone observed after dietary repeated exposure in the rat, mouse and dog

Species	Sex	Duration of exposure	Dose at which effects become adverse (mg/kg bw/day)	Increase in relative weight at this dose (%)	Hepatocellular hypertrophy	Other adverse histopathological or biochemical findings
Rat	Male	28 days	182	15.5	3/5	None
Rat	Female	28 days	193	17	4/5	None
Rat	Male	90 days	505	37	10/10	↑ cholesterol, protein and calcium
Rat*	**Female**	**90 days**	**150**	**17**	**1/10**	**↑ cholesterol, protein and calcium**
Rat	Male F_0	2-generations (reproductive)	140	19	None	None
Rat	Female F_0	2-generations (reproductive)	187	21	18/25	None
Rat	Male F_1	2-generations (reproductive)	140	14	None	None
Rat	Female F_1	2-generations (reproductive)	187	21	20/25	None
Mouse	*Male*	*28 days*	*> 985*	*13*	*4/5*	*None*
Mouse	Female	28 days	984	21.5	2/5	None
Mouse	Male	90 days	930	23	10/10	None
Mouse*	**Female**	**90 days**	**583**	**17.5**	**3/9**	**None**
Dog	Male	90 days	750	20	2/4	None
Dog*	**Female**	**90 days**	**100**	**22**	**None**	**None**
Dog	Male	12 months	> 500	10	None	None
Dog	Female	12 months	> 500	10	None	None

* Lowest dose identified in the species for adverse liver effects

The kidney was also identified as a clear target organ in rats and dogs (but not in mice); increased kidney weights were observed in rats and dogs, with the rat being the more sensitive species and the male the more sensitive sex.

<u>Rat</u>

In the rat, the main target organs of toxicity identified were the liver and kidney. Additional effects were seen in the thyroid, prostate and uterus.

Adverse effects on the liver

Adverse increased liver weights (> 15 % compared to controls, with or without hepatocellular hypertrophy) were seen from 182 / 193 mg/kg bw/day (males / females) in the 28-day study ([redacted] (2015a)) and from 150 mg/kg bw/day (females) in the 90-day study ([redacted] (2016a)). In addition, similar liver effects

were seen from ≈ 180 / 220 mg/kg bw/d (mean dose males / females) in the 2-generation study ([redacted] (2016c)) and at the top dose of 217 / 176 mg/kg bw/day (males / females) in the 2-year carcinogenicity study ([redacted] 2017) These liver effects were associated with alterations of some clinical-chemistry parameters indicative of liver toxicity (e.g. increased cholesterol, BUN, triglycerides) from 379 mg/kg bw/day (females) in the 28-day study, from 150 mg/kg bw/day (females) in the 90-day study and at the top-dose of 167 mg/kg bw/day in females in the 2-year carcinogenicity study.

Adverse effects on the kidney

Regarding adverse effects on the kidney, there were no histopathological or biochemical signs of adversity related to the kidney; however increased weights (absolute & relative to body weights > 10 % compared to control groups) indicative of an adverse effect were noted after 90 days' exposure from 121 mg/kg bw/day in males and 351 mg/kg bw/day in females; it was also noted that the relative weights remained high following 28 days of recovery. The effects on kidney weights after 28-days exposure were less pronounced. Treatment-related and adverse changes in kidney weights relative to body weight were also noted in the 2-generation reproductive toxicity study at the top dose in the F_0 generation (141 / 261 mg/kg bw males / females) and in the F_1 generation (140 / 187 mg/kg bw males / females). In contrast to these short-term studies, there were no adverse effects noted for the kidneys (including kidney weights) in the 2-year carcinogenicity study at weeks 52 and 104 in both sexes up to the top-dose of 217 / 167 mg/kg bw/day (males / females). Overall, there were consistent adverse effects on kidney weights in both sexes in the rat short-term studies.

Other findings

Mild follicular cell hypertrophy of the thyroid was observed at the top dose of 505 / 351 mg/kg bw/day (males / females) in the 90-day study without associated changes in thyroid weights; no such finding was seen following a recovery period of 28 days. However there were no clear thyroid weight or histopathology changes noted in the 28-day study or the 2-year year carcinogenicity study. Females showed a slight increase in the incidence of follicular cell adenoma (benign tumours) in the thyroid gland at the top-dose of 167 mg/kg bw/day however these tumour findings were regarded as chance findings unrelated to treatment (B.6.5.1). Overall there were no clear adverse effects on the thyroid in the rat following repeated administration of bixlozone.

In addition, there was increased prostate inflammation at the top dose of 140 mg/kg bw/d in the rat 2-generation study. The toxicological significance of this finding on reproductive organs is discussed further in the summary of the reproductive toxicity section.

Furthermore, systemic toxicity characterised by decrease in body weight and/or body weight gain were observed in females from 193 mg/kg bw/day in the 28-day study (and at 740 mg/kg bw/day for males), at the top-dose of 351 / 505 mg/kg bw/day (males / females) in the 90-day study, at the top dose of 167 / 217 mg/kg bw/day in the 2-year carcinogenicity study and at the top dose in the F_0 generation (141 / 261 mg/kg bw males / females) and in the F_1 generation (140 / 187 mg/kg bw males / females) in the 2-generation reproductive toxicity study.

Female rats were more sensitive than males; this is consistent with the indication that females are more highly exposed to bixlozone than males since parallel toxicokinetics investigations showed higher concentrations of bixlozone in females' blood compared to males.

<u>Mouse</u>

In the mouse, the main target organ of toxicity was the liver. There were no adverse effects noted on the thyroid. Additional effects on kidney, epididymes and stomach were noted following chronic exposure.

Adverse effects on the liver

Increased liver weights with associated histopathology (enlarged individual hepatocytes with expanded eosinophilic cytoplasm) were seen from the top-dose of 984 mg/kg bw/day (females only) in the 28-day study ([redacted] (2015b)), 930 / 583 mg/kg bw/day (males / females) in the 90-day study ([redacted] (2016b)), and 647 / 834 mg/kg bw/day (males / females) in the 18-month carcinogenicity study ([redacted] (2017)). These effects were only associated with alterations of some clinical-chemistry parameters indicative of liver toxicity (e.g. increased ALT) at the top dose of 985 mg/kg bw/day (males) in the 28-day study. The adverse effects on the liver seen in the mouse occur at higher dose levels than the adverse effects observed in the rat.

Other findings

On chronic exposure, decreased sperm in the epididymes and chronic inflammation of the glandular stomach were seen in males from the mid dose of 126 mg/kg bw/day (1000 ppm), with kidney pelvis dilation noted in males at the top dose of 647 mg/kg bw/day (5000 ppm). Despite the uncertainties in these findings (sex-

specificity, low biological plausibility), no robust argumentations (including appropriate HCD) have been provided by the applicant to discount their toxicological significance. The relevance of the reduced epididymal sperm counts observed in the 18-month chronic study is discussed further in the summary of the reproductive toxicity section.

In addition to the toxic effects seen in the liver and these other organs, decreases in body weight and/or body weight gain were observed in females only at the top-dose of 1384 mg/kg bw/day in the 28-day study and in the carcinogenicity study at the top dose of 834 mg/kg bw/day.

Dog

In the dog, the main target organ of toxicity identified was the liver. Additional effects were seen in the prostate and WBC.

Adverse effects on the liver

Regarding adverse effects seen in the liver, increased absolute and relative liver weights to body weight with associated hepatocellular hypertrophy was observed in both sexes from 370 / 309 mg/kg bw/day (males / females) in the 28-day (oral, dietary) range-finding study ([redacted] (2016b)). In the following 90-day study ([redacted] (2017)), the method of oral administration was changed from dietary to capsule owing to palatability issues noted in the 7-day ([redacted] (2015c)) and 28-day studies; in this study increased absolute and relative liver weights to body weight were seen from 100 mg/kg bw/day in females and at the top-dose of 750 mg/kg bw/day in males, accompanied with minimal hepatocellular hypertrophy in males only. However, no liver-related adverse effects were noted in the 12-month (oral, capsule) study up to the top dose of 500 mg/kg bw/day. Overall the dog appears to be relatively less sensitive to the toxic effect of bixlozone on the liver compared to the rat.

Other findings

Thyroid weight was increased in at the top dose of 750 mg/kg bw/day in females and from 300 mg/kg bw/day in males in the 90-day study, but no associated histopathology was seen. The thyroid was not affected in the 28-day study up to the top dose of approx. 1340/1080 mg/kg bw/day (M/F) or in the 1-year study up to 500 mg/kg bw/day. It is most likely these changes in thyroid weight are a spurious finding.

Changes in kidney weights were seen from 38 mg/kg bw/day in males in the dietary 28-day study. However, these changes were not reproduced after 90 days (up to 750 mg/kg bw/day) or 1 year treatment (up to 500 mg/kg bw/day) using capsules. It is possible that the kidney weight changes seen in the 28-day study were the consequence of the method of administration (dietary vs capsules) and associated severe toxicity due to palatability problems rather than the test substance itself. In addition, in the absence of any associated histopathology or changes in clinical-chemistry and urinalysis parameters indicative of kidney toxicity, these kidney weight changes are regarded as spurious findings.

Haematological changes (such as WBC, PT, LUC and lymphocytes absolute) were also observed in females at the top-dose of 750 mg/kg bw/day in the 90-day study and in males at 500 mg/kg bw/day in the 12-month study.

Reductions in prostate weight with associated immaturity were seen in the 90-day study from 300 mg/kg bw/day, but not up to 500 mg/kg bw/day in the 1-year study. On this basis, these prostate findings are considered to be of minimal toxicological significance. The prostate findings in the dog are discussed further in the summary of the reproductive toxicity section.

In addition to toxic effects noted in the organs above, body weight and body weights gain were severely affected in dogs after 28 days' dietary exposure due to palatability issue with the test substance. Hence the mode of administration of bixlozone for the 90-day and 12-month studies was changed from dietary to capsule; following this change there was no effects seen in body weight or the body weight gain at any dose tested for both sexes.

A table summarising the main adverse effects observed in the repeated-dose toxicity studies of bixlozone is presented below:

Table B 6.3.6.2: Summary of repeated-dose toxicity of bixlozone

Study, guideline, reference Acceptability	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
Dietary 7-day **Rat** (Crl :CD9(SD), males & females, 5/sex/group) Not to GLP Not to OECD Guideline Deviations: None F9600 technical, batch PL13-0385 Purity: 99.2% ([redacted]) (2015d) ***Supplementary***	0, 4000, 7000 and 12000 ppm Equivalent to : Males: 0, 441, 698 and 1067 mg/kg bw/day Females: 0, 434, 763 and 1250 mg/kg bw/day	No robust NOAEL can be derived from this non-GLP, non-OECD compliant study. *< 4000 ppm (441 / 434 mg/kg bw/day males / females)* Based on relative liver weight increases > 15 % in both sexes observed in both sexes at 4000 ppm	N.A.	There were no reported deaths; **12000 ppm** ↓ body weight, body weight gain and/or food consumption (both sexes) ↑ relative liver weight > 15 % in both sexes **7000 ppm** ↓ body weight, body weight gain and/or food consumption (M) ↑ relative liver weight > 15 % in both sexes **4000 ppm** ↑ relative liver weight > 15 % in both sexes

Study, guideline, reference Acceptability	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
Dietary 7-day **Mouse** (Crl:CD-1(ICR)), males & females, 5/sex/group) Not to GLP Not to OECD Guideline Deviations: None F9600 technical, batch PL13-0385 Purity: 99.2% ([redacted], (2015c) ***Supplementary***	0, 2000, 4000 and 6000 ppm Equivalent to : Males: 0, 404, 960 and 1348 mg/kg bw/day Females: 0, 476, 886 and 1460 mg/kg bw/day	No robust NOAEL can be derived from this non-GLP, non-OECD compliant study. *< 2000 ppm (404 / 476 mg/kg bw/day males / females)* Based on relative liver weight increases > 15 % in both sexes and reduced body weight / gain observed in both sexes at 4000 ppm	N.A.	There were no reported deaths or clinical signs of toxicity. **6000 ppm** ↓ body weight gain (F): 42 % ↓ body weight (M): 11 % ↑ relative liver weight > 15 % in both sexes **4000 ppm** ↑ relative liver weight > 15% in both sexes **2000 ppm** ↑ relative liver weight > 15% in both sexes
Dietary 7-day **Dog** (Beagle), males & females, 2/sex/group) Not to GLP Not to OECD Guideline Deviations: None F9600 technical, batch PL13-0385 Purity: 99.2% ([redacted], (2015e) ***Supplementary***	0, 2500, 5000, 10000 and 30000 ppm Equivalent to : Males: 0, 67, 185, 292 and 818 mg/kg bw/day Females: 0, 79, 187, 244 and 716 mg/kg bw/day	No robust NOAEL can be derived from this non-GLP, non-OECD compliant study. *10000 ppm (292 / 244 mg/kg bw/day males / females)*	N.A.	**30000 ppm** ↓ body weight & food consumption (first 3 days). **2500, 5000, 10000 ppm** No adverse effects observed.
Oral (capsule) 7-day **Dog** (Beagle), males & females, 2/sex/group) Not to GLP Not to OECD Guideline Deviations: None F9600 technical, batch PL14-0049 Purity: 96.0% ([redacted],	0, 150, 350 and 550 mg/kg bw/day	*> 550 (males / females)* No robust NOAEL can be derived from this non-GLP, non-OECD compliant study.	N.A.	No treatment-related findings were observed during the study period up to the highest dose tested.

Study, guideline, reference Acceptability	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
(2016b) ***Supplementary***				
28 day, dietary **Rat,** Crl :CD9(SD), males & females, 5/sex/toxicology group (Inc. control), 9/sex/toxicokinetic group (3/sex/control group) GLP OECD 407 (2008) Deviations : None F9600 technical, batch PL13-0385 Purity: 99.2% [redacted] (2015a) ***Acceptable***	0, 750, 2500, 5000, and 10000 ppm (for toxicology and toxicokinetic groups) Equivalent to : Males (M): 0, 57, 182, 359 and 740 mg/kg bw/d Females (F): 0, 61, 193, 379 & 733 mg/kg bw/d	57 / 61 (M/F) (750 ppm) (The applicant proposed a NOAEL of 5000ppm)	180 / 193 (M/F) (2500 ppm)	There were no deaths or clinical signs of toxicity **<u>10000 ppm (740 / 733 mg/kg bw/day M / F)</u>** ↓ body weight (F): 18 %** ↓ body weight gain: 59 %** (F) & 14 % (M) ↓ food consumption (F): 41 %** (days 0-7), 17 %** (days 7-14) and 22 %** (days 14-27) ↓ food consumption (M): 20 % (days 0-7)** *Organ weights* ↑ absolute liver weights: 32 %** (F) & 56 %** (M) ↑ relative liver weights: 61 %** (F) & 65.5 %** (M) ↑ relative kidney weights: 14** % (F & M) *Histopathology - liver* Hepatocellular hypertrophy: 5/5 mild (F) & 4/5 mild + 1/5 moderate (M) *Clinical chemistry* ↑ total protein (9 % F &13 %** M), ↑ albumin (11 % M**), ↑ globulin (12 % M** & 15 %* F),↑ cholesterol (79 %** M & 91 %** F), ↑ BUN (45.5 %** F), ↑ triglyceride (86 %* F) **<u>5000 ppm (359 / 379 mg/kg bw/day M / F)</u>** ↓ food consumption (F): 23.5 %** (days 0-7) & 17 %* (days 7-21) ↓ food consumption (M): 16 %* (days 0-7) *Organ weights* ↑ absolute liver weight: 19 %* (F) ↑ relative liver weight: 29 %** (F) & 23 %** (M) *Histopathology - liver*

Study, guideline, reference Acceptability	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
				Hepatocellular hypertrophy: 1/5 minimal & 4/5 mild (F); 3/5 minimal & 2/5 mild (M) *Clinical chemistry* ↑ cholesterol (43 %* F) **2500 ppm (182 / 193 mg/kg bw/day M / F)** ↓ food consumption in females: 12 %** (days 0-7) & 11 %* (days 7-14) *Organ weights* ↑ relative liver weight: 17 %** (F), 15.5 %** (M) *Histopathology - liver* Hepatocellular hypertrophy: 4/5 minimal (F) & 3/5 minimal (M) **750 ppm (57 / 61 mg/kg bw/day M / F)** No treatment-related findings.
28 day, dietary **Mouse,** Crl:CD-1, males & females, 5/sex/group GLP OECD 407 (2008) Deviations: none F9600 Technical, batch PL13-0385 Purity: 99.2% [redacted] (2015b) ***Acceptable***	0, 1000, 2000, 4000, and 5000 ppm Equivalent to: Males: 0, 187, 381, 788 & 985 mg/kg bw/day Females: 0, 289, 554, 984 & 1384 mg/kg bw/day	554 mg/kg bw/day females (2000 ppm) (The applicant proposed a NOAEL of 5000ppm based on absence of adverse findings)	788/984 mg/kg bw/day (M/F) (4000 ppm)	There were no treatment-related deaths. **5000 ppm (985 / 1384 mg/kg bw/day M / F)** ↓ body weight gain: 19 % (F) *Organ weights* ↑ absolute liver weight: 15 % (F) & 14 % (M) ↑ relative liver weight: 24 %** (F) & 13 %* (M) *Histopathology - liver* Hepatocellular hypertrophy: 3/5 F (2 minimal, 1 mild) & 4/5 M (2 minimal, 2 mild) *Clinical chemistry* ↑ ALT: 137 %* (M) **4000 ppm (788 / 984 mg/kg bw/day M / F)** *Organ weights* ↑ absolute liver weight: 18 %* (F) ↑ relative liver weight: 21.5 %** (F) *Histopathology - liver* Hepatocellular hypertrophy: 2/5 F (minimal) & 1/5 M (minimal) **2000 ppm (381 / 554 mg/kg bw/day M / F) & 1000 ppm (187 / 289 mg/kg bw/day M / F)** No treatment-related findings.
28 day, dietary **Dog**, Beagle, males & females,	0, 1000, 3000, 10000 & 30000 ppm Equivalent to	No robust NOAEL and LOAEL could be set from	N.A.	There were no treatment related deaths No statistical analysis was performed **30000 ppm (≈ 1015 / 1110 mg/kg bw/day**

Study, guideline, reference Acceptability	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
2/sex/group Bixlozone technical, batch PL14-0049 Purity: 96% Vehicle: acetone GLP Dose-range finding study (loosely follows OECD 409) [redacted] (2016b) ***Supplementary***	control, 1000, 3000, and 10000 ppm groups: Males: 0, 38, 134 & 370 mg/kg bw/d Females: 0, 39, 108 & 309 mg/kg bw/d (test substance intake for 30000 ppm males and females could not be accurately calculated due to food supplementation)	this study as severe toxicity was seen as consequence of method of administration and palatability issues		**<u>M / F)</u>** Clinical signs: thin body condition (1 M), ↓ defecation (2 M) ↓ body weight: 17 % (M) and 9 % (F) ↓ body-weight gain: 116 % (M) and 90 % (F) ↓ food consumption led to food supplementation (M & F) *Organ weights* ↑ relative liver weight: 80 % (F) and 53 % (M) ↑ absolute liver weight: 30 % (M) and 63.5 % (F) ↑ relative kidney: 41 % (M) and 40 % (F) ↑ absolute kidney weight: 20 % (M) and 28 % (F) *Histopathology - liver* Hepatocellular hypertrophy in 2 / 2 M (1 minimal & 1 mild) Hepatocellular hypertrophy in 2/ 2 F (mild) **<u>10000 ppm</u>** ↓ body-weight gain: 17 % (M) and 54 % (F) ↓ food consumption in M & F ↑ Relative liver weight: 28.5 % (F) and 20 % (M) ↑ absolute liver weight: 19 % (M) and 21 % (F) ↑ kidney weight in M: 22 % absolute and 23 % relative Hepatocellular hypertrophy in 2 / 2 M (minimal) Hepatocellular hypertrophy in 2 / 2 F (minimal) **<u>3000 ppm</u>** Relative liver weight: 15 % (F) 1000 ppm ↓ body weight gain: 45.5 % (F) **<u>1000 ppm (38 / 39 mg/kg bw/day M / F)</u>** No treatment-related findings.
90-day, dietary (Includes neurotoxicity and recovery phase) **Rat,** Crl :CD9(SD), males & females,	0, 500, 2000, and 8000 ppm (males) Equivalent to: 0, 29, 121 & 505 mg/kg bw/day	29/37 (M/F) (500 ppm) (Applicant also proposed a NOAEL of	121/150 (M/F) (2000 ppm)	One male (5000 ppm) was found dead on day 87 (undetermined cause); there were no clinical signs of toxicity at any dose. **<u>8000 / 5000 ppm M/F (505 / 351 mg/kg bw/day M / F)</u>** <u>1 death (M)</u>

Study, guideline, reference Acceptability	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
21/sex/group or 16/sex group (including neurotoxicity phase) F9600 technical, batch PL14-0049 Purity: 96% Vehicle: acetone GLP OECD 408 (1998) & OECD 424 (1997) Deviations: None [redacted] (2016a) ***Acceptable***	(males) 0, 500, 2000, and 5000 ppm (females) Equivalent to: 0, 37, 150 & 351 mg/kg bw/day 90-days continuous dosing Recovery period: 28-days (5/sex group)	2000 ppm)		↓ body weight: 9 %* (M) & 9.5 %** (F) ↓ body weight gain: 18 %** (M) & 23 %** (F) food efficiency in M: - 14 %** (main group) & + 22 % (recovery group) food efficiency in F: - 11 %* (main group) & + 55 % (recovery group) *Organ weights* ↑ liver weights in M: 21.5 %** (absolute) & 37 %** (relative) ↑ liver weights in F: 22.5 %** (absolute) & 34 %** (relative) ↑ kidney weights in F: 17 %** (relative) *Histopathology - liver* Hepatocellular hypertrophy: 10/10 M (1 minimal, 6 mild, 3 moderate) and in 10/10 F (7 mild, 3 moderate) Macrovascular vacuolation 5/10 M (4 minimal, 1 moderate) *Histopathology - thyroid* Follicular cell hypertrophy (mild): 3/10 M & 5/10 F *Clinical chemistry* ↑ Cholesterol 40.5 %** (F) & 77 %** (M) ↑ globulin +11 %* and calcium +4.5 %* (F) **28-day recovery group (8000 / 5000 ppm)** ↑ food consumption 11 %** (M) & 4.5 % (F) ↑ relative liver weight 10 % (M) ↑ relative kidney weight 22 %** (M) Mild macro vascular vacuolation in liver 1/5 (M) ↑ cholesterol 31 %** (F) **2000 ppm (121 / 150 mg/kg bw/day M / F)** *Organ weights* ↑ liver weights in females: 16 %* absolute & 17 %** relative ↑ kidney weights in males: 15 %* (absolute) & 14.5 %** (relative) *Histopathology - liver* Hepatocellular hypertrophy 1/10 F (mild) *Clinical chemistry* ↑ cholesterol +44 %**, globulin +11 % and calcium +4.5 %* (F)

Study, guideline, reference Acceptability	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
				500 ppm (29 / 37 mg/kg bw/day M / F) No treatment-related findings.
90 day, dietary **Mouse**, Crl:CD1(ICR), males & females, 10/sex/ toxicology group, 12/sex/toxicokinetic group F9600 technical, batch PL14-0049 Purity: 96% Vehicle: acetone GLP OECD 408 (1998) Deviations: none [redacted] (2016b) ***Acceptable***	0, 1000, 2250, and 5000 ppm Equivalent to: Males: 0, 180, 414 & 930 mg/kg bw/day Females: 0, 257, 583 & 1185 mg/kg bw/day	257 (F) (1000 ppm) (The applicant proposed a NOAEL of 5000 ppm)	583 (F) (2250 ppm)	There were no test-substance related deaths or clinical signs of toxicity **5000 ppm (930 / 1185 mg/kg bw/day M/F)** ↑ relative liver weights: 23 % (M) & 21 % (F) ↑ absolute liver weights: 23 % (M) & 20 % (F) Hepatocellular hypertrophy in 10/10 M (1 minimal, 9 mild) Hepatocellular hypertrophy in 3/9 F (1 minimal, 2 mild) **2250 ppm (414 / 583 mg/kg bw/day M/F)** ↑ relative liver weights: 17.5 % (F) ↑ absolute liver weights: 13 % (F) Hepatocellular hypertrophy in 4/10 M (3 minimal, 1 mild) Hepatocellular hypertrophy in 3/9 F (minimal) **1000 ppm (180 / 257 mg/kg bw/day M/F)** No adverse effects observed **Neurotoxicity (90 days)** A higher incidence of alert females was noted at week 12 from the mid-dose of 2000 ppm (150 mg/kg bw/day), but, in isolation, this finding is not considered to represent a specific neurotoxic response. Please refer to Section **Error! Reference source not found.** for more details.
90 day, capsule **Dogs**, Beagle, males & females, 4/sex/group F9600 technical, batch PL14-0049 Purity: 96 % Vehicle: none GLP OECD 409 (1998) [redacted] (2016c)	0, 30, 100, 300, and 750 mg/kg/day	30 (F) (The applicant proposed a NOAEL of 750 mg/kg bw/day)	100 (F)	There were no treatment-related deaths or clinical signs of toxicity; there was no effect on body weight or food consumption at any dose levels. **750 mg/kg bw/day** *Organ weights* ↑ absolute liver weights: 54 %** (F) & 21 % (M) ↑ relative liver weights: 46 %** (F) & 20 %** (M) ↑ relative thyroid weight: 54 % (F) & 21 % (M) ↓ prostate weight: absolute 41 % and relative

Study, guideline, reference Acceptability	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
Acceptable				43 % and associated immaturity *Histopathology - liver* Hepatocellular hypertrophy in 2/4 males (minimal) *Clinical chemistry* ↑ WBC (37 %* wk. 6), ↑ lymphocytes (43 %* wk. 6 & 39 %* wk. 12/13), ↑ LUC (+150 % wk 6) in F **300mg/kg bw/day** *Organ weights* ↑ relative liver weight: 21.5 %** (F) ↓ abs (19%) and rel (26.5%) prostate weight and associated immaturity **100 mg/kg bw/day** *Organ weights* ↑ liver weights in F (27 %* absolute, 22 %** relative) **30 mg/kg bw/day** No treatment-related findings.
12 months, capsule **Dogs,** Beagle, males & females, 4/sex/group F9600 technical, batch PL14-0049 Purity: 96% Vehicle: none GLP OECD 409 (1998) Deviations: none [redacted] (2017) ***Acceptable***	0, 20, 100, and 500 mg/kg/day	100 (M) (The applicant proposed a NOAEL of 500 mg/kg bw/day)	500 (M)	There were no treatment-related deaths or clinical signs of toxicity; there was no effect on body weight or food consumption at any dose levels. **500 mg/kg bw/day** ↑ WBC (+35 % week 26*; +27 % week 52) ↑ monocyte absolute (+55% week 26*;+15 % week 52) ↑ lymphocytes absolute (+34 % week 26; +55 % week 52**) ↑ PT (+8 % week 26*; +13 % week 52**) in males **100 & 20 mg/kg bw/day** No adverse effects observed.
21 day, dermal **Rat**, Crl :CD9(SD), males & females, 10/sex/group GLP OECD 410 (1981) Deviations : None F9600 technical, batch PL14-0049 Purity: 99.2% [redacted] (2016)	0, 100, 300, and 1000 mg/kg bw/day	> 1000 mg/kg bw/day based on no adverse effects observed at the highest dose tested (The applicant proposed a NOAEL of 1000 mg/kg bw/day)	> 1000 mg/kg bw/day	There were no deaths or clinical signs of toxicity **1000, 300 and 100 mg/kg bw/day** No adverse effects observed.

Study, guideline, reference Acceptability	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
Acceptable				

Consideration of the classification of bixlozone for STOT-RE and setting of the overall/most sensitive NOAEL for short-term toxicity

The table below presents all the relevant NOAEL and LOAEL values identified in the available short-term studies. The overall / most relevant **NOAEL for short-term toxicity** is proposed to be set from the 90-day oral (dietary) repeated-dose toxicity conducted in the rat at **29 and 37 mg/kg bw/day** in males and females respectively (with a respective LOAEL of 121 / 150 mg/kg bw/day in males / females based on treatment-related and adverse increase in liver weights accompanied by increased cholesterol, protein and calcium and 1/10 hepatocellular hypertrophy in females and kidney weights in males). This NOAEL is consistent with the NOAEL of **30 mg/kg bw/day** from the 90-day dog study.

When compared with the classification criteria for STOT-RE, the liver and kidney were clear target organs at doses above the cut-off values for classification into category 2 for the oral route of exposure in the rat (10 < dose ≤ 100 mg/kg bw/day). Therefore, HSE concludes that bixlozone should not be classified for STOT-RE 2 according to Regulation (EC) N°1272/2008 (see MCL report for further details).

Overall, the repeated-dose toxicity of bixlozone has been adequately investigated in studies in rats, mice and dogs; the critical target organ was identified as the liver followed by the kidney, and adverse effects observed in these organs could be relevant to humans. Classification for repeated-dose toxicity according to Regulation (EC) N°1272/2008 is not warranted (for more details please see aligned MCL Report).

Table B.6.3.6.3. Summary of NOAEL values for the short-term toxicity of bixlozone

Study, guideline, reference, acceptability	Species, doses tested	NOAEL mg/kg bw/d	LOAEL mg/kg bw/d	*Adverse* effects at LOAEL
Dietary 28-day GLP OECD 407 (2008) Deviations: None ([redacted](2015a)) **Acceptable**	**Rat** (Crl :CD9(SD), males & females) F9600 technical; Batch PL13-0385 Purity 99.2% 0, 750, 2500, 5000, and 10000 ppm Equivalent to : Males: 0, 57, 182, 359 and 740	57 (males) 61 (females) (750 ppm) (The applicant proposed a NOAEL of 740 / 733 for males / females)	182 (males) 193 (females) (2500 ppm)	↑ relative liver weight > 15% in both sexes Hepatocellular hypertrophy: 4/5 minimal (F) & 3/5 minimal (M) Adverse ↓ on body weight, body weight gain and food consumption in females

Study, guideline, reference, acceptability	Species, doses tested	NOAEL mg/kg bw/d	LOAEL mg/kg bw/d	*Adverse* effects at LOAEL
	mg/kg bw/day Females: 0, 61, 193, 379 & 733 mg/kg bw/day			
Dietary 28-day GLP OECD 407 (2008) Deviations: none ([redacted] (2015b)) **Acceptable**	**Mouse** (Crl:CD-1, males & females) F9600 technical; Batch PL13-0385 Purity 99.2% 0, 1000, 2000, 4000, and 5000 ppm Equivalent to: Males: 0, 187, 381, 788 & 985 mg/kg bw/day Females: 0, 289, 554, 984 & 1384 mg/kg bw/day	381 (males) 554 (females) (2000 ppm) (The applicant proposed a NOAEL of 985 / 1384 mg/kg bw/day for males / females)	788 (males) 984 (females) (4000 ppm)	↑ relative liver weight: 21.5 % (females) ↑ absolute liver weight: 18 % (females) Hepatocellular hypertrophy: 2/5 females (minimal) & 1/5 males (minimal)
Dietary 28-day Range-finding study GLP OECD 409 (1998) Deviations: Due to palatability issue at 30000 ppm animals were fed with food supplementation thus the mean achieved bixlozone consumption could not be calculated accurately ([redacted] (2016b)) **Supplementary**	**Dog** (Beagle) F9600 Technical; batch PL14-0049 Purity 96 % 0, 1000, 3000, 10000, and 30000 ppm Equivalent to control, 1000, 3000, and 10000 ppm groups: Males: 0, 38, 134 & 370 mg/kg bw/day Females: 0, 39, 108 & 309 mg/kg bw/day (test substance intake for 30000 ppm males and females could not be accurately calculated due to food supplementation)	No robust NOAEL and LOAEL could be set from this study as severe toxicity was seen as consequence of method of administration and palatability issues	39 mg/kg bw/day (females) (1000 ppm)	↓ body weight gain: 45.5 % (females)
Dietary 90-day GLP OECD 408 (1998) & OECD 424 (1997) Deviations: None ([redacted] (2016a))	**Rat** (Rat, Crl :CD9(SD), males & females) F9600 Technical; batch PL14-0049 Purity 96 % 0, 500, 2000, and 8000 ppm (males)	**29** (males) **37** (females) (500 ppm) (The applicant proposed a NOAEL of 121 / 150 for males / females)	121 (males) 150 (females) (2000 ppm)	↑ liver weight > 15% in females (16 % absolute & 17 % relative) ↑ kidney weights in males (15 % absolute &14.5 % relative) Hepatocellular hypertrophy 1/10 females (mild) ↑ cholesterol, protein and calcium in females

Study, guideline, reference, acceptability	Species, doses tested	NOAEL mg/kg bw/d	LOAEL mg/kg bw/d	*Adverse* effects at LOAEL
Acceptable	Equivalent to: 0, 29, 121 & 505 mg/kg bw/day 0, 500, 2000, and 5000 ppm (females) Equivalent to: 0, 37, 150 & 351 mg/kg bw/day			
Dietary 90-day GLP OECD 408 (1998) Deviations: None ([redacted] (2016a)) **Acceptable**	**Mouse** (Crl:CD1(ICR), males & females) F9600 Technical; batch PL14-0049 Purity 96 % 0, 1000, 2250, and 5000 ppm Equivalent to: Males: 0, 180, 414 &930 mg/kg bw/day Females: 0, 257, 583 & 1185 mg/kg bw/day	180 (males) 257 (females) (1000 ppm) (The applicant proposed a NOAEL of 930 / 1185mg/kg bw/day for males / females)	414 (males) 583 (females) (2250 ppm)	↑ relative liver weights: 17.5 % (females) ↑ absolute liver weights: 13 % (females) Hepatocellular hypertrophy in 4/10 males (3 minimal, 1 mild) Hepatocellular hypertrophy in 3/9 females (minimal)
90-day, capsule GLP OECD 409 (1998) Deviations: None ([redacted], 2015) **Acceptable**	**Dog** (Beagle, males and females) F9600 technical; batch PL14-0049 Purity 96 % 0, 30, 100, 300, and 750 mg/kg/day	**30** (females) (The applicant proposed a NOAEL of 750 mg/kg bw/day)	100 (females)	↑ liver weights in females (27 % absolute, 22 % relative) There were no treatment-related deaths or clinical signs of toxicity; there was no effect on body weight or food consumption at any dose levels.
12-month, capsule GLP OECD 452 (1998) Deviations: None ([redacted] (2017)) **Acceptable**	**Dog**, (Beagle, males & females), 4/sex/group F9600 technical, batch PL14-0049 Purity: 96% 0, 20, 100 and 500 mg/kg bw/day	100 (The applicant proposed a NOAEL of 500 mg/kg bw/day)	500	↑ WBC, monocyte absolute, lymphocytes absolute & PT (males)
21-day, dermal GLP OECD 410 (1981) Deviations: None ([redacted] (2016)) **Acceptable**	**Rat**, Crl :CD9(SD), males & females F9600 technical, batch PL14-0049 Purity: 99.2% 0, 100, 300 and 1000 mg/kg bw/day	> 1000 (The applicant proposed a NOAEL of 1000 mg/kg bw/day)	N/A	No adverse effects observed up to the highest dose tested

Study, guideline, reference, acceptability	Species, doses tested	NOAEL mg/kg bw/d	LOAEL mg/kg bw/d	*Adverse* effects at LOAEL

B.6.4. GENOTOXICITY

The genotoxic potential of bixlozone was tested both *in vitro* and *in vivo*, in a range of modern (2018) genotoxicity assays, conducted in accordance with relevant OECD test guidelines and in compliance with GLP. The available *in vitro* studies are a bacterial reverse mutation assay (Ames test), a mammalian chromosomal aberration assay using Chinese hamster ovary (CHO-K1) cells and a mammalian cell gene mutation assay (Mouse Lymphoma Assay). The available *in vivo* study is a mammalian erythrocyte micronucleus test in the rat.

B.6.4.1. In vitro studies

B.6.4.1.1. *Ames test*

Study	Bacterial Reverse Mutation Assay with F9600 Technical
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	Bruce, S. (2018)
Date performed	August 2017
Test facility	BioReliance Corporation, Rockville, MD
Report reference	Study no. BioReliance AE80XH.503.BTL
Guideline(s)	OECD Guideline 471 (1997)
Deviations from the guideline	None of significance
GLP	Yes
Test material	F9600 Technical Batch: JB-F9600-201603004 Purity: 96.82 %
Study acceptable	Yes

Material and Methods

The potential of bixlozone to induce gene mutations in bacteria was investigated in a GLP compliant Ames test with an initial toxicity-mutation test (Experiment 1) and a confirmatory test (Experiment 2) using *S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and the WP2 *uvr*A strain of *E. coli* . The assays were performed with and without metabolic activation from Aroclor 1254-induced rat liver S9, using the plate incorporation methodology. All test items, including controls, were tested in duplicate. Bixlozone was tested at concentrations of 1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate in the initial toxicity-mutation test and in triplicate at 5, 15, 50, 150, 500, 1500 and 5000 µg per plate for the confirmatory test. Dimethyl sulfoxide (DMSO) was used as the vehicle control. A validated method of analysis for the test substance concentrations is not required for this study.

Results

Initial toxicity-mutation test (Experiment 1)

Precipitation of the test item was observed at 5000 µg per plate under all conditions. Cytotoxicity, defined as reduction in background lawn or reduction in revertant count, was observed at 5000 µg per plate in all strains.

No positive mutagenic responses were observed, in either the presence or absence of metabolic activation, up to the limit of cytotoxicity and / or precipitation. The reference mutagens used as positive controls in the test produced a distinct increase in revertant colonies and those values were within the HCD provided by the laboratory. Vehicle controls values were also within the HCD provided.

Based upon these results, the concentrations tested in the confirmatory mutagenicity test were set at 5, 15, 50, 150, 500, 1500 and 5000 µg per plate.

Table B 6.4.1.1:Summary of the initial toxicity-mutation test (Experiment 1)

Treatment	Dose (µg/plate)	S9	Revertant colonies/plate[a]									
			TA98		TA100		TA1535		TA1537		WP2 *uvr*A	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
F9600 Technical	5000	-	7	4	0	0	0	0	0	0	10	0
	1500	-	10	2	0	0	0	0	8	4	11	1
	500	-	12	4	89	6	13	2	11	1	15	1
	150	-	14	2	86	2	12	3	4	4	21	4
	50	-	13	4	89	9	11	1	6	1	16	1
	15	-	11	1	82	11	14	2	8	2	21	1
	5	-	11	6	89	7	8	4	6	1	17	2
	1.5	-	12	1	68	5	12	4	8	1	18	5
DMSO	50 µL	-	11	6	97	11	13	4	6	1	18	2
Positive [b]		-	86	32	649	184	629	1	479	28	341	31
F9600 Technical	5000	+	15	2	0	0	0	0	0	0	9	1
	1500	+	17	1	88	6	8	1	10	1	24	5
	500	+	16	3	94	13	18	3	6	1	28	4
	150	+	19	4	105	8	9	4	8	4	22	6
	50	+	17	1	102	9	11	1	11	0	26	8
	15	+	22	2	114	24	10	1	8	5	36	4
	5	+	19	5	97	16	14	7	8	2	25	4
	1.5	+	21	5	105	6	9	0	9	0	30	0
DMSO	50 µL	+	18	0	96	11	16	5	8	1	29	3
Positive [c]		+	273	4	696	11	77	8	40	11	350	40

a Replicates per dose n=2

b TA98: 2-nitrofluorene at 1 µg/plate, TA100 and TA1535: sodium azide at 1 µg/plate, TA1537: 2-nitrofluorene at 75 µg/plate, WP2 *uvr*A: methyl methane sulfonate at 1000 µg/plate

c TA98 and TA1535: 2-aminoanthracene (2AA) at 1 µg/plate, TA100 and TA1537: 2AA at 2 µg/plate, WP2 *uvr*A: 2AA at 15 µg/plate

Confirmatory mutagenicity test (Experiment 2)

Precipitation of the test item was observed at 5000 µg per plate under all conditions. Cytotoxicity, defined as reduction in background lawn or reduction in revertant count, was observed from 1500 or at 5000 µg per plate under most conditions.

No positive mutagenic responses were observed in either the presence or absence of metabolic activation up to the limit of cytotoxicity and / or solubility. The reference mutagens used as positive controls produced a distinct increase in revertant colonies and values were within the HCD provided by the laboratory. Negative control values were also within the HCD provided. Based on the response produced by the positive and vehicle controls, the validity of the study was confirmed.

Overall, there was no evidence of a mutagenic potential of bixlozone in this study.

Table B 6.4.1.2: Summary of the confirmatory mutation test (Experiment 2)

Treatment	Dose (µg/plate)	S9	Revertant colonies/plate[a]									
			TA98		TA100		TA1535		TA1537		WP2 *uvr*A	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
F9600 Technical	5000	-	12	1	0	0	0	0	2	1	20	1
	1500	-	13	2	0	0	0	0	5	1	23	4
	500	-	16	3	86	5	13	2	5	1	23	3
	150	-	16	5	96	19	13	4	6	2	21	3
	50	-	16	5	84	6	15	5	6	1	23	2
	15	-	13	4	87	13	12	5	6	2	24	4
	5	-	15	3	85	12	13	2	8	3	27	4
DMSO	50 µL	-	14	2	81	15	12	3	6	2	25	4
Positive [b]		-	104	24	638	165	691	202	458	133	349	29
F9600 Technical	5000	+	18	2	0	0	0	0	5	2	13	5
	1500	+	24	3	89	11	9	1	6	1	21	1
	500	+	23	2	102	17	10	1	7	2	25	5
	150	+	21	2	110	10	11	5	8	2	26	7
	50	+	22	3	96	19	10	4	7	2	27	3
	15	+	22	2	118	13	12	3	7	2	22	1
	5	+	25	1	112	11	14	4	8	2	29	6
DMSO	50 µL	+	27	4	104	8	13	2	8	1	25	7
Positive [c]		+	361	58	1110	441	69	7	52	13	243	63

a Replicates per dose n=3
b TA98: 2-nitrofluorene at 1 µg/plate, TA100 and TA1535: sodium azide at 1 µg/plate, TA1537: 2-nitrofluorene at 75 µg/plate, WP2 *uvr*A: methyl methane sulfonate at 1000 µg/plate
c TA98 and TA1535: 2-aminoanthracene (2AA) at 1 µg/plate, TA100 and TA1537: 2AA at 2 µg/plate, WP2 *uvr*A: 2AA at 15 µg/plate

Conclusion

The results from this study shows that bixlozone does not have the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system up to the limit of cytotoxicity and / or solubility.

Overall, there was no evidence of a mutagenic potential of bixlozone in this study.

Bruce, S. (2018)

B.6.4.1.2. *In vitro mammalian chromosomal aberration assay*

Study	*In Vitro* Mammalian Chromosomal Aberration Assay in Chinese Hamster Ovary (CHO) Cells with F9600 Technical
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	Roy, S. (2018)
Date performed	July 2017
Test facility	BioReliance Corporation, Rockville, MD
Report reference	Study no. BioReliance AE80XH.331.BTL
Guideline(s)	OECD Guideline 473 (2016)
Deviations from the guideline	None
GLP	Yes
Test material	F9600 Technical Batch: JB-F9600-201603004 Purity: 96.82 %
Study acceptable	Yes

Material and Methods

The potential for bixlozone to induce structural chromosomal aberrations was tested *in vitro* in a chromosomal aberration assay using Chinese hamster ovary (CHO-K_1) cells, in both the absence and presence of metabolic activation (Aroclor 1254-induced rat liver S9). The study was conducted in compliance with GLP and in accordance with OECD 473 (2016). CHO cells were treated for 4 hours in the absence and presence of S9, and for 20 hours in the absence of S9. DMSO was used as the vehicle control and the positive controls chosen were mitomycin C (MMC) for the treatment without S9 and cyclophosphamide (CP) for the treatments with S9.

Excessive cytotoxicity was defined as ≥ 50 % reduction in cell growth index, relative to the vehicle control. Cell growth was determined by Relative Increase in Cell Counts (RICC) as a measure of cytotoxicity as described in the OECD Guideline 473 (below). The cell counts and percent viability were used to determine cell growth inhibition (CGI) relative to the vehicle control (% cytotoxicity = 100 – RICC).

$$\text{RICC (\%)} = \frac{\text{(Increase in number of cells in treated cultures (final starting))}}{\text{(Increase in number of cells in control cultures (final starting))}} \times 100$$

In the preliminary toxicity assay (single culture / concentration), the concentrations tested ranged from 0.2 to 2000 µg/mL, which is the limit concentration recommended in the OECD Guideline. Concentrations of 20, 40, 80, 100, 120, 140, 160, 180 µg/mL were used for all three conditions in the main experiment in duplicate cultures. A validated method of analysis for the test substance concentrations is not required for this study.

Results

Preliminary toxicity assay

Cytotoxicity (defined as ≥ 50 % CGI, relative to the vehicle control) was observed at concentrations ≥ 200 µg/mL. By the end of the treatment period, visible precipitate was observed at concentrations ≥ 600 µg/mL in all three test conditions. Based on the findings of the preliminary assay, concentrations of 20, 40, 80, 100, 120, 140, 160, 180 µg/mL were used for all three tests in the main experiment.

Main chromosome aberration assay (Table B 6.4.1.3 & Table B 6.4.1.4)

In the main experiment, cytotoxicity was observed at concentrations ≥ 160 µg/mL in the 4 hour treatment without S9, at concentrations ≥ 140 µg/mL in the 4 hour treatment + S9, and at concentrations ≥ 80 µg/mL in the 20 hour treatment condition without S9. Hence the concentrations selected for the evaluation of chromosomal aberrations in CHO cells were 80, 120, and 160 µg/mL for the 4 hour treatment without S9; 40, 80, and 140 µg/mL for the 4 hour treatment with S9; and 20, 40, and 80 µg/mL for the 20 hour treatment condition without S9.

Table B 6.4.1.3: Cell growth and mitotic inhibition observed in the main chromosome aberration assay

Treatment Condition	Treatment Time	Highest evaluated dose (µg/mL)	Cell growth inhibition (%)	Mitotic inhibition (%)
Without S9	4 hr	160	50	11
	20 hr	80	60	38
S9-activated	4 hr	140	57	41

In the 4 and 20 hour tests without S9, no statistically significant or dose-dependent increases in structural aberrations were observed at any concentrations. However, following the 4 hour treatment with S9 a dose-dependent increase in chromosomal and chromatid structural aberrations was observed. The increase in chromosomal aberrations was statistically significant at the top concentration of 140 µg/mL (12.7 % aberrant cells vs 1.7 % in negative control, $p \leq 0.01$ Fisher's exact test and $p \leq 0.05$ Cochran-Armitage test) and above the historical 95 % control value (0.00 % to 3.88 %). It is noted that cytotoxicity is extensive at this concentration (47 % from control); the OECD Guideline 473 paragraph 22 states that "care should be taken in interpreting positive results only to be found in the higher end of cytotoxicity 55 +/-5 %.

All controls fulfilled the requirements for a valid test as described in the study report and in the OECD Guideline 473.

Overall, under the conditions of this study, there was evidence of a clastogenic potential of bixlozone *in vitro*, in the presence of metabolic activation at the top concentration of 140 µg/mL at which cytotoxicity occurred, making the interpretation of the positive result uncertain.

Table B 6.4.1.4: Summary results from the main chromosome aberration assay (n = 2)

Treatment condition	Test Substance	Concentration (µg/mL)	Cytotoxicity (% from control)[a]	Aberrations per Cell[b,d] Mean ± SD	Aberrant Cells		Carrying exchanges	Total polyploid cells (Mean %)[e]
					Numerical (Mean %)[b]	Structural (Mean %)[c]		
4-h +16 h Recovery Without S9	DMSO	NA	-	0.013 ± 0.115	1.3	1.3	0	1.3
	F9600 Technical	80	-6	0.010 ± 0.100	1.7	1.0	0	1.3
		120	26	0.023 ± 0.151	2.0	2.3	0	2.0
		160	50	0.017 ± 0.128	1.7	1.7	0.5	1.7
4-h +16 h Recovery With S9	DMSO	NA	-	0.017 ± 0.0128	2.7	1.7	0	2.0
	F9600 Technical	40	7	0.010 ± 0.100	2.7	1.0	0.5	1.3
		80	33	0.033 ± 0.180	2.7	3.3	1	2.0
		140	57	0.133 ± 0.360	3.0	**12.7****	2.5	2.0
	CP	5	47	0.247 ± 0.491	2.3	**22.0****	6	2.3
20-h Without S9	DMSO	NA	-	0.013 ± 0.115	1.7	1.3	0.5	1.7
	F9600 Technical	20	20	0.007 ± 0.082	3.0	0.7	0	2.7
		40	44	0.010 ± 0.100	2.7	1.0	0	2.7
		80	60	0.010 ± 0.100	1.3	1.0	0	1.3
	MMC	0.1	11	0.200 ± 0.505	0.7	**17.3****	8.5	0.7

DMSO: Dimethyl sulfoxide; MMC: Mitomycin C; CP: Cyclophosphamide; NA: Not Applicable; Fisher's Exact Test: ** $p \leq 0.01$.
a. Based on cell growth inhibition relative to solvent control.
b. Includes polyploid and endo-reduplicated cells.
c.. Does not include cells with only gaps
d. Severely damaged cells counted as 10 aberrations.
e. Does not include endo-reduplicated cell.
f. SD = Standard Deviation.

Conclusion

Under the conditions of this GLP and OECD compliant *in vitro* mammalian chromosomal aberration test using CHO cells bixlozone caused an increase in structural chromosomal aberrations in the S9-activated test system after a 4 hour exposure. Overall, under the conditions of this study, there is evidence that bixlozone is clastogenic *in vitro* at the top concentration of 140 µg/mL, at which extensive cytotoxicity occurred.

Since the interpretation of positive results found in the higher end of cytotoxicity 55 +/-5 % should be taken with care, further testing is required. An *in vivo* micronucleus test is recommended by the Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Journal 2011;9(9):2379) as a follow-up for substances found to be clastogens or aneugens *in vitro* and has been provided by the applicant. The study is presented in Section B.6.4.2).

Roy, S. (2018)

B.6.4.1.3. *In Vitro Mammalian Cell Gene Mutation Test*

Study	*In Vitro* Mammalian Cell Gene Mutation Test (L5178Y/TK+/- Mouse Lymphoma Assay) with F9600 Technical
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	Dutta, A. (2018)
Date performed	January 2018
Test facility	BioReliance Corporation, Rockville, MD
Report reference	Study no. BioReliance AE80XH.704.BTL
Guideline(s)	OECD Guideline 490 (2016)
Deviations from the guideline	None of significance
GLP	Yes
Test material	F9600 Technical Batch: JB-F9600-201603004 Purity: 96.82 %
Study acceptable	Yes

Material and Methods

The mutagenic potential of bixlozone was assessed in a GLP and OECD compliant mammalian cell gene mutation assay (L5178Y/TK+/- Mouse Lymphoma Assay).

In a preliminary toxicity assay, cultures of L5178Y/TK+/- cells were treated in duplicate for 4 hours with concentrations of 7.81, 15.6, 31.3, 62.5, 125, 250, 500, 1000 and 2000 µg/mL bixlozone, both in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9). For the main experiment, cultures of L5178Y/TK+/- cells were treated in triplicate for 4 or 24 hours with without S9 and 4 hours with S9 with concentrations of bixlozone indicated in Table B.6.4.1.3.1 and then sub-cultured using soft agar to allow phenotypic expression prior to mutant selection. A validated method of analysis for the test substance concentrations is not required for this study.

The positive controls used in this study were methyl methane sulfonate (MMS) for treatments without S9, and 7,12-Dimethyl-benz(a)anthracene (DMBA) for treatments with S9; DMSO was used as the vehicle control. The laboratory applied the criteria for the determination of a valid test recommended in the OECD Guideline.

Results

Preliminary toxicity assay

The Relative Suspension Growth (RSG) determining cytotoxicity in the three tests was 42, 55 and 14 % at concentrations of 125 µg/mL in all treatment conditions. RSG was 0 % in all tests at concentrations higher than 125 µg/mL. Based upon these results, the concentrations chosen for the main assay were:

Table B 6.4.1.5: Concentrations selected for the main MLA

Treatment Condition	Treatment Time	Concentrations (µg/mL)
Non-activated	4 hours	7.81, 15.6, 31.3, 62.5, 125, 175, 200 and 250
	24 hours	7.81, 15.6, 31.3, 62.5, 125, 175 and 200
S9-activated	4 hours	7.81, 15.6, 31.3, 62.5, 125, 175, 200 and 250

Main Mouse Lymphoma Assay (Table B 6.4.1.6)

Visible precipitate was observed at the concentration of 250 µg/mL at the beginning of treatment in all tests. The test substance did not have an adverse impact on the pH of the cultures (pH was 7.5 at the top concentrations).

In the 4 hour test with S9 at concentrations of 7.81, 15.6, 31.3, 62.5 and 125 µg/mL and in the 24 hour test at concentrations of 7.81, 15.6, 31.3 and 62.5µg/mL, RSG ranged from 89-18 % and 93-30 % respectively and were thus sub-cultured to allow phenotypic expression prior to mutant selection. In the 4 hour test with S9 one replicate at 125 µg/mL exhibited excessive cytotoxicity. The relative total growth of the cultures was compliant

with the OECD Guideline recommendations and ranged from 16 to 77 % (4 hour treatment with S9) and 28 to 109 % (24 hour treatment without S9).

There was no dose-related or statistically significant increase in mutant frequency observed following 4-hour treatment with S9 and 24-hour treatment without S9 at any tested concentration; the results did not meet the criteria for a positive response according to the laboratory and the OECD criteria for a positive response. The positive and negative controls were acceptable according to the OECD Guideline criteria

In the 4 hour test without S9 the mutant frequency of the positive control did not meet the criteria for an acceptable positive control thus this treatment condition was repeated in a confirmatory experiment. The concentration levels selected for the retest were 15.6, 31.3, 62.5, 125, 150 and 200 µg/mL. No visible precipitate was observed at the beginning or end of treatment. The test substance did not have an adverse impact on the pH of the cultures (pH was 7.5 at the top concentration). The cultures treated at 15.6, 31.3, 62.5, 125 and 150 µg/mL showed a RSG ranging from 87 % to14 % and were sub-cultured to allow phenotypic expression prior to mutant selection. The relative total growth of the cultures ranged from 14 to 93 % and were thus within the recommended OECD Guideline values.

There was no dose-related or statistically significant increase in mutant frequency observed following 4-hour treatment without S9; the results did not meet the criteria for a positive response according to the laboratory and the OECD criteria for a positive response. The positive and negative controls were acceptable according to the OECD Guideline criteria

Table B 6.4.1.6: Summary of the Mouse Lymphoma Assay results (including the confirmatory results for the 4 hours treatment without S9)

Exposure Time	S9	Substance	Concentration (µg/mL)	Relative Total Growth (% of Control)	Mutant Frequency (per 10^6 cells)	Induced Mutant Frequency (per 10^6 cells)
4 Hours	-	DMSO	-	100	50	NA
			-		31	
		MMS*	20	36	227	186
			15	56	139	99
4 Hours (confirmatory)	-	DMSO	-	100	68	NA
			-		58	
		F9600 Technical	15.6	77	60	-3
			15.6	85	52	-11
			31.3	78	52	-11
			31.3	93	61	-2
			62.5	45	80	17
			62.5	69	53	-9
			125	34	71	8
			125	29	82	19
			150	14	59	-4
			150	20	41	-22
			200	+	+	+
			200	+	+	+
		MMS	20	7	676	613
			15	16	523	460

Exposure Time	S9	Substance	Concentration (µg/mL)	Relative Total Growth (% of Control)	Mutant Frequency (per 10^6 cells)	Induced Mutant Frequency (per 10^6 cells)
4 Hours	+	DMSO	-	100	66	NA
			--		73	
		F9600 Technical	7.81	77	58	-12
			7.81	74	59	-11
			15.6	53	84	14
			15.6	60	64	-5
			31.3	51	76	7
			31.3	42	82	12
			62.5	42	90	20
			62.5	34	107	37
			125	16	118	48
			125	+	+	+
			175	+	+	+
			175	+	+	+
			200	+	+	+
			200	+	+	+
			250	+	+	+
			250	+	+	+
		DMBA	1.5	13	466	396
			1.0	28	397	327
24 Hours	-	DMSO	-	100	46	NA
			-		56	
		F9600 Technical	7.81	83	55	4
			7.81	109	47	-4
			15.6	79	52	1
			15.6	87	58	7
			31.3	54	49	-2
			31.3	55	50	-1
			62.5	28	59	8
			62.5	38	60	9
			125	+	+	+
			125	+	+	+
			175	+	+	+
			175	+	+	+
			200	+	+	+
			200	+	+	+
		MMS	7.5	20	403	352
			5	35	327	276

+= Plates were not sub-cultured due to excessive toxicity (Only one culture for 125µg/mL (4-hour treatment with S9) was sub-cultured as the other culture with 125µg/mL had excessive toxicity for evaluation)

* The mutant frequency of the positive control did not meet the criteria for an acceptable positive control thus this treatment condition was repeated in a confirmatory experiment

DMSO = dimethyl sulfoxide; DMBA = 7,12-Dimethyl-benz(a)anthracene; MMS = methyl methane sulfonate; NA = Not Applicable

Conclusion

Under the conditions of this GLP and OECD compliant *in vitro* mammalian cell gene mutation assay (L5178Y/TK+/- Mouse Lymphoma Assay), bixlozone did not induce forward mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells, either in the presence or absence of metabolic activation, up to cytotoxic concentrations.

In conclusion, there was no evidence of a mutagenic potential of bixlozone in this study.

Dutta, A. (2018)

B.6.4.2. *In vivo* studies in somatic cells

An *in vivo* micronucleus test conducted in the rat is available as a follow-up for the positive results obtained in the *in vitro* chromosome aberration test (Roy. S, 2018). This is in line with the recommendations laid out in the Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Journal 2011;9(9):2379), where the conduct of an *in vivo* micronucleus test is recommended as a follow-up for substances found to be clastogens or aneugens *in vitro*.

Moreover, a study which demonstrated systemic and bone marrow exposure to bixlozone at the doses used in this *in vivo* rat bone marrow micronucleus assay was conducted and is described in detail in Section B.6.1.1.4.

Study	*In Vivo* Mammalian Erythrocyte Micronucleus Assay in Rats with F9600 Technical
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2018)
Date performed	Oct 2017
Test facility	[redacted]
Report reference	Study no. [redacted] AE80XH.125M021.
Guideline(s)	OECD Guideline 474 (2016)
Deviations from the guideline	None
GLP	Yes
Test material	F9600 Technical Batch: JB-F9600-201603004 Purity: 96.82 %
Method of analysis	Not required for this study.
Study acceptable	Yes

Material and Methods

Bixlozone was tested for its clastogenic activity and/or disruption of the mitotic apparatus *in vivo* in a mammalian erythrocyte micronucleus assay in the rat. The test substance vehicle was 0.5 % (w/v) CMC (400 cPs) in 5 % (w/v) Tween® 80 in deionized water. Doses of 0, 500, 1000 or 2000 mg/kg bw were administered to 5 males per group by oral gavage on two consecutive days approximately 24 hours apart in the main experiment. The doses were chosen upon toxicological information provided by the applicant to the laboratory, and an additional 2 animals were dosed at 2000 mg/kg bw to cover for any possible mortality.

24 hours after the second dose, the animals were euthanised and bone marrow collected and processed for the scoring of 4000 Polychromatic (immature) Erythrocytes (PCEs) per animal for the presence of Micronucleated Polychromatic Erythrocytes (MnPCEs). Positive control slides (fixed and unstained) generated from a recent study were included to verify scoring. These slides were generated from male rats treated once with cyclophosphamide monohydrate (CP) at 40 mg/kg, and the bone marrow harvested 24 hours after treatment. The positive control induced a detectable and statistically significant increase in the incidence of MnPCEs (Student's t-test, $p \leq 0.05$) and the values were within the HCD provided by the laboratory (dates from 2012-2015, n = 749).

At least 500 total erythrocytes (PCEs + NCEs) were scored per animal to determine the proportion of PCEs as an index of bone marrow cytotoxicity. Moreover bone marrow exposure to bixlozone was demonstrated at the doses used in this study and is described in detail in Section B.6.1.1.4.

Results

Clinical findings and body weight gain ***(Error! Reference source not found.)***

No mortality occurred at any dose level during the course of the definitive assay. Clinical signs were seen in a dose-related manner: piloerection occurred in animals treated at 500 and 1000 mg/kg bw whilst piloerection, hunched position and diarrhoea were noted in animals treated at the maximum dose of 2000 mg/kg bw. There was also a dose-dependent decrease in body weight gain observed in the treated animals compared to the controls.

Overall there are indications of a dose-related systemic exposure to bixlozone and/or its metabolites.

Table B 6.4.2.1:Summary of the clinical findings in the mammalian erythrocyte micronucleus assay

Treatment	Observation	Number of animals with clinical signs/Number of surviving animals							Number of animals found dead/ Total number of animals dosed	% change in mean body weight from day 1 to day 3
		Day 1			Day 2			Day 3		
			Post-dose (hours)			Post-dose (hours)				
		Pre-dose	+ 1	+ 2	Pre-dose	+ 1	+ 2			
Vehicle 0 mg/kg	Normal	5/5	5/5	5/5	5/5	5/5	5/5	5/5	0/5	+ 5.3
F9600 Technical 500 mg/kg	Normal Piloerection	5/5 0/5	5/5 0/5	5/5 0/5	5/5 0/5	3/5 2/5	3/5 2/5	5/5 0/5	0/5	+ 4.6
F9600 Technical 1000 mg/kg	Normal Piloerection	5/5 0/5	5/5 0/5	3/5 2/5	5/5 0/5	5/5 0/5	4/5 1/5	3/5 2/5	0/5	+ 1.3
F9600 Technical 2000 mg/kg	Normal Piloerection Hunched Position Diarrhoea	7/7 0/7 0/7 0/7	7/7 0/7 0/7 0/7	4/7 3/7 0/7 0/7	7/7 0/7 0/7 0/7	1/7 6/7 2/7 1/7	2/7 5/7 2/7 3/7	4/7 2/7 1/7 0/7	0/7	- 0.1

Bone marrow analysis – scoring (Table B 6.4.2.2)

There was a statistically significant decrease (- 5 %) in the ratio of immature to mature erythrocytes observed at 2000 mg/kg/day dose group compared to the negative control group, indicating the test substance induced bone marrow toxicity. However, this reduction in this ratio did not exceed the OECD recommended value of – 20 % of the vehicle control proportion. Exposure of the bone marrow to the test item is further supported by the fact that kinetic studies in the rat showed that the substance and/or its metabolites were systemically available and reached the bone marrow (please refer to Section B.6.1. for more details from the study conducted by [redacted] (2017f)).

Overall, it can be concluded that the bone marrow was exposed to the test item, and therefore the study is capable of assessing the genotoxic potential of bixlozone.

There was no statistically significant increase in the incidence of MnPCEs in the treated groups, when compared to the negative control group (ANOVA followed by Dunnett's post-hoc analysis, $p > 0.05$). The number of MnPCEs in the negative control groups did not exceed the historical control range provided by the laboratory (dates from 2012-2015, n = 1047). The positive control slides (fixed and unstained) generated from a recent study and included to verify scoring showed a statistically significant increase in the incidence of MnPCEs compared to the vehicle control (Student's t-test, $p \leq 0.05$) and the values were within the HCD provided by the laboratory.

Overall, there was no evidence of a clastogenic or aneugenic effect of bixlozone in this study.

Table B 6.4.2.2: Summary of the mammalian erythrocyte micronucleus assay results in rats

Treatment	Dose (mg/kg/day)	No. of Animals/ Group	% PCE (mean ± SD)	% change in % PCE compared to control	% MnPCE (mean ± SD)	MnPCE / PCE Scored
Vehicle control	0	5	52.7 ± 1.0	-	0.08 ± 0.02	16 / 20000
F9600 Technical	500	5	52.1 ± 0.4	-1	0.07 ± 0.02	13 / 20000
	1000	5	51.8 ± 0.7	-2	0.09 ± 0.02	17 / 20000
	2000	5	**50.1 ± 1.3****	-5	0.09 ± 0.02	18 / 20000
CP	40	5	**43.2 ± 3.1****	-18	**2.48 ± 0.21****	495 / 20000

*p < 0.05 or **p < 0.01, One-Way ANOVA with Post-Hoc Dunnett's Test or T-Test
24 Hrs MnPCE Male GLM P-value = 0 269, R-sqr = 21.21%

Conclusion

In a GLP and OECD guideline compliant *in vivo* micronucleus study, bixlozone did not cause an increase in the incidence of MnPCEs in the bone marrow of male rats administered the test substance up to the limit dose of 2000 mg/kg bw. Exposure of the bone marrow to the test item was demonstrated in an ADME study presented in Section B.6.1.1.4. Thus, it can be concluded that bixlozone is not clastogenic or aneugenic in a valid *in vivo* rat micronucleus assay.

[redacted] (2018)

B.6.4.3. *In vivo* studies in germ cells

As bixlozone was negative *in vivo* in a mutagenicity test in somatic cells, no *in vivo* mutagenicity studies in germ cells were conducted or were considered required.

B.6.4.4. Summary of genotoxicity

The genotoxic potential of bixlozone was tested *in vitro* in a bacterial reverse mutation assay (Ames test), an *in vitro* chromosome aberration study using CHO cells, an *in vitro* mammalian cell gene mutation test (L5178Y/TK+/- Mouse Lymphoma Assay); and *in vivo* a rat micronucleus study was also conducted. The studies were all performed according to the relevant OECD TGs and were GLP compliant.

Bixlozone did not induce gene mutations in bacteria or mouse lymphoma cells *in vitro* but was clastogenic *in vitro* with metabolic activation (S9) at an exposure leading to significant cytotoxicity. However, when tested *in vivo* in a valid rat bone marrow micronucleus study up to the limit dose of 2000 mg/kg bw, the clastogenic activity seen *in vitro* was not evident *in vivo*. At this dose level, clinical signs of toxicity, reduction in body weight gain and toxicokinetic data all confirm exposure to the bone marrow occurred; these confirm optimal assay conditions were met. Nor was there any evidence of aneugenicity in the rat bone marrow micronucleus study.

Overall, it is concluded that bixlozone is not genotoxic *in vivo* and the data requirements of Regulation 283/2013 have been met. Therefore, classification of bixlozone for mutagenicity is not warranted (see also aligned MCL report).

A summary of all the available genotoxicity studies is shown in the table below.

Table B 6.4.4.1 Summary of genotoxicity studies with bixlozone

Study	Concentrations of Substance tested	Result	Reference
***In vitro* assays**			
Ames test OECD N° 471 (1997) GLP: yes	0, 5, 15, 50, 150, 500, 1500 and 5000 µg / plate ± S9	Negative	Bruce, S. (2018)
Chromosomal aberrations study in CHO cells OECD N° 473 (2016) GLP: yes	0, 20, 40, 80, 100, 120, 140, 160, 180 µg/mL used for all three treatment conditions in the main experiment. Treatments: 4-h ± S9; 20-h - S9	Clastogenic following 4-h treatment with S9	Roy, S. (2018)
L5178Y/TK+/- Mouse Lymphoma cells mutagenicity study OECD N° 490 (2016) GLP: yes	0, 7.81, 15.6, 31.3, 62.5, 125, 175, 200 and 250 µg/mL 4-h treatment + S9 0, 15.6, 31.3, 62.5, 125, 150 and 200 µg/mL 4-h treatment - S9 0, 7.81, 15.6, 31.3, 62.5, 125, 175 and 200 µg/mL 24-h treatment – S9	Negative	Dutta, A. (2018)
***In vivo* assay**			
Rat micronucleus assay *in vivo* (oral gavage) OECD N° 474 (2016) GLP: yes	0, 500, 1000 and 2000 mg/kg bw/day Treatment on two consecutive days 24-h apart	Negative	[redacted] (2018)

B.6.5. LONG-TERM TOXICITY AND CARCINOGENESIS

The carcinogenic potential of bixlozone has been investigated in a 2-year combined chronic toxicity/carcinogenicity study in rats and an 18-month carcinogenicity study in mice via the dietary route.

Toxicokinetic parameters have been included in these studies; short summaries of the toxicokinetic findings are presented in this Section but for further details please refer to Section 6.1. of the DAR. Analyses were conducted using a validated high performance liquid chromatography method using UHPLC-MS/MS detection. The method validation is within a separate study (Lucarell, 2016a; WIL Research, USA; Study No. WIL-105112) and the data are presented in Volume 3, Section CA B.4, Point CA 4.1.2(c).

B.6.5.1. 2-year combined chronic toxicity / carcinogenicity study in rats

Study	A 2-Year Oral (Dietary) Combined Chronic Toxicity and Carcinogenicity Study with Toxicokinetic Measurements of F9600 Technical in Sprague Dawley Rats.
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2017)
Date performed	Oct 2014 – Nov 2017
Test facility	[redacted]
Report reference	Study no. [redacted]-105121
Guideline(s)	OECD Guideline 453 (2009)
Deviations from the guideline	The study was conducted following the version of the OECD 453 i.e. adopted in 2009. The guideline has since been updated in June 2018. Changes from the 2009 version are minor.
GLP	Yes
Test material	F9600 technical Batch PL14-0049, purity 96 %
Method of analysis	[redacted], *2014;* [redacted]*; Study No.* [redacted]*-105110) and the data are presented in Volume CA B.4, Point CA 4.1.2(c)* *The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to the lack of explanation of the use of a non-linear calibration.* However, the method is considered sufficient for regulatory purposes. It is further noted that the dosage concentrations used in this study were 250, 1000, and 5000/3000 (males/females) ppm which is within the validation dataset and therefore appropriate.
Study acceptable	Yes

Material and Methods

In a GLP- and OECD-compliant combined chronic toxicity / carcinogenicity study, bixlozone technical was administered *ad libitum* in the diet of Crl:CD (SD) rats for 52 and 104 consecutive weeks for the chronic toxicity and carcinogenicity groups respectively.

The substance dose levels were selected from the previous 28- and 90-day oral dietary studies in rats ([redacted] 2015 and [redacted] 2016, respectively). It was anticipated that in this study the high dose of 5000 ppm and 3000 ppm for males and females, respectively, would be, or approach the Maximum Tolerated Dose (MTD) based on the findings of the 90-day rat study. The high dose selected here represents the geometric means between the high- and mid-doses used in the 90-day rat study (Table B.6.5.5.1). The different high-dose for males and females is based on findings of toxicity and toxicokinetic differences between male and female rats observed in the 28-day and 90-day studies.

Administered doses were 250, 1000, and 5000/3000 ppm (males/females), equating to consumed levels of 10, 41, and 217 mg/kg bw/day respectively for males and 13, 53, and 167 mg/kg bw/day respectively for females. The females treated at the highest dose were originally treated with 4000 ppm however at Day 49 (week 7), the dose administered was reduced to 3000 ppm based on severe adverse effects (body weight loss approaching 10 %, corresponding lower mean body weight gains by 18-20 % and lower mean food consumption by 9-12 %).

Table B 6.5.1.1: Mean test substance intake in the rat chronic/carcinogenicity study with bixlozone – comparison with repeated dose toxicity

	Males			Females		
Dose (ppm)	250	1000	5000	250	1000	4000/3000
Dose (mg/kg bw/d) Carcinogenicity phase (2-years)	10	41	217	13	53	167
Dose (mg/kg bw/d) Repeated dose toxicity – 90 days	29	121	505	37	150	351

Each chronic toxicity/carcinogenicity group consisted of 60 animals/sex. Ten animals/sex/group were assigned to the interim necropsy (chronic toxicity, 52 weeks). The remaining 50 animals/sex/group were assigned to the primary necropsy (carcinogenicity, 104 weeks). In addition, 10 animals/sex from the same shipment of animals were assigned to sentinel groups to provide biological samples for diagnosis of potential disease conditions. These animals were housed in the same room as the animals assigned to the main study but were not treated.

The toxicokinetic groups were treated for 182 days and each toxicokinetic group consisted of 4 animals/sex. For the toxicokinetic evaluation, blood samples were collected from 4 animals/sex/toxicokinetic group on Day 1, 14, 28, 90, and 182. All surviving toxicokinetic animals were euthanised following the final blood collection (Day 182) and the liver collected to measure its weight and for possible future histopathological evaluation.

The incidence of tumours was analysed by Peto's mortality-prevalence method (Peto *et al.*, 1980).

Results

Deaths

There were no test substance-related deaths during the chronic toxicity phase (12 months) and the carcinogenicity phase (24 months). For the first 12-months (chronic phase) mortality was low overall (< 10 % in all groups) and was similar across control and treated groups (Table B.6.5.5.2); however, mortality was much higher (54-76 %) at 24 months across all groups, including controls. No explanation in the study report has been provided for the high mortality rate observed in all groups at the end of the 2 years.

The most common causes of death in males were pituitary adenoma and undetermined in all groups, including controls. In females the most common causes of death were pituitary neoplasms (adenoma and carcinoma combined) and mammary gland neoplasms (adenoma, adenocarcinoma and fibroadenoma combined) in all groups. The causes of death in all animals were commonly noted in Sprague Dawley rats (according to the extended historical control database provided by the applicant), were equally seen in concurrent controls and did not show a dose-response relationship.

Survival rates (Table B 6.5.1.2 & Table B 6.5.1.3)

Overall percent survival at the end of the study was low: for males at week 104, (all causes of death combined), survival rate was 30 % (15/50), 46 % (23/50), 42 % (21/50), and 46 % (23/50) for the control, 250, 1000, and 5000 ppm group, respectively. For females, the overall percent survival was 27 % (13/50), 34 % (17/50), 26 % (13/50), and 24 % (12/50) for the control, 250, 1000, and 3000 ppm group, respectively.

The current OECD Guideline 453 (2018) refers to the OECD guidance document 116 on the conduct and design of chronic toxicity and carcinogenicity studies supporting test guidelines 451, 452 and 453 (ENV/JM/MONO(2011)47) for the consideration of survival rates. The guidance document 116 recommends that survival should ideally be no less than 50 % in all groups by 24 months in rats for a negative result in a carcinogenicity study (paragraphs 162-163). It is also noted that the US EPA Health Effects Test Guidelines 870.4300 for combined chronic toxicity/carcinogenicity studies (US EPA 712–C–98–212, 1998) specify that for these studies, survival in any group should not fall below 50 % at 18 months and 25 % at 24 months in the rat.

The survival rates retrieved at 104 weeks in this present study are thus lower than the recommended 50 % in OECD guidance document 116 but within the rates of 25 % recommended by the US EPA. Moreover, it is noted that the survival rates at 18 months were still relatively high and ranging from 60 up to 78 % while at 21 months, for all groups excepted for the female group treated at 1000 ppm (34 % survival), survival was still around or above 50 %. Therefore, the survival data at 21 months are in agreement with the recommendation of the OECD guidance document 116 and overall, HSE considers that the power of the study is not compromised and that the study is acceptable for investigating the potential of bixlozone to cause chronic toxicity and cancer.

Table B 6.5.1.2: Survival rates in the rat carcinogenicity study over 2 years (104 weeks)[a]

Dose level (ppm)	Males				Females			
	0	250	1000	5000	0	250	1000	3000
Chronic Phase (52 weeks)								
Initial N (including satellite group)	60	60	60	60	60	60	60	60
Killed for humane reasons	2	2	0	2	2	2	2	2
Found dead	3	1	1	1	0		0	2
Died during anaesthesia								
Accidental trauma	0	0	0	0	1	0	0	0
Total deaths after 52 weeks	5	3	1	3	3	2	2	4
% survival after 52 weeks (/60)	**92**	**95**	**98**	**95**	**97[a]**	**97**	**97**	**93**
Carcinogenic Phase (104 weeks)								
Initial N	50	50	50	50	50	50	50	50
Killed for humane reasons	24	20	13	20	27	28	26	28
Found dead	12	7	16	7	9	5	11	10
Died during anaesthesia								
Accidental trauma	0	0	0	0	1	0	0	0
Total deaths after 18 months	11	16	12	11	17	20	14	12
% survival after 18 months (week 80) / 50	**78**	**68**	**76**	**78**	**67**	**60**	**72**	**76**
Total deaths after 21 months	23	20	19	17	24	27	33	25
% survival after 21 months (week 91) / 50	**54**	**60**	**62**	**66**	**52**	**46**	**34**	**50**
Total deaths after 104 weeks	36	27	29	27	37	33	37	38
Scheduled euthanasia; primary necropsy / 50	15	23	21	23	13	17	13	12
% survival after 104 weeks / 50	**30**	**46**	**42**	**46**	**27**	**34**	**26**	**24**

[a] survival corrected for accidental deaths
[b] includes found death and partially cannibalised animals

Despite the terminal low survival rates across all groups, there was no effect of treatment on mortality or survival. Kaplan-Meier estimates (Kaplan and Meier, 1958) of group survival rates were calculated, by sex, and showed no statistical difference in the survival between animal groups of same sex with overall p values well above statistically significance.

Table B 6.5.1.3: Kaplan-Meier estimates of survival and p values

		Kaplan-Meier estimates (%) and p-values					
Sex	**Week**	**0 ppm**	**250 ppm**	**1000 ppm**	**5000 ppm**	**Overall**	**Trend**
M	**52**	92	95	98	95		
	80	79	69	76	79		
	104	31	46	42	46		
	P value (p)		NT	NT	NT	0.4417	0.1421
F	**52**	97	97	97	93		
	80	68	60	73	77		
	104	27	34	26	24		
	P value (p)		NT	NT	NT	0.9359	0.6243

p-values (p): Comparisons using vehicle group
* = Statistically significant.
NA = Not applicable.
NT = Not tested.

Clinical findings

Test substance-related clinical observations noted throughout the study were limited to the top dose groups, with yellow material on various body surfaces (urogenital area and ventral trunk) in males and dermal atonia and thin body condition in females. Dermal atonia and thin body condition are considered adverse by HSE.

Palpable masses (Table B 6.5.1.4)

The number of multiple masses, the mean number of masses per animal, and the mean number of days to the first mass in the treated groups were generally similar to or lower than those in the control group. There were no statistically significant differences when the control and treated groups were compared, with the exception of the top dose males where an increase in the number of animals with palpable masses compared to controls was seen. The nature of the palpable masses identified is discussed further below in the sub-section for "*Histopathology -Neoplastic findings*" where skin tumours were identified, particularly in top-dose males.

Table B 6.5.1.4: Summary of palpable mass in the two-year rat study with bixlozone

	Males				Females			
Dose (ppm)	**0**	**250**	**1000**	**5000**	**0**	**250**	**1000**	**4000/3000**
Dose (mg/kg bw/day)	**0**	**10**	**41**	**217**	**0**	**13**	**53**	**167**
Number of animals in dose group	60	60	60	60	60	60	60	60
Number of animals with masses	7	17	8	14	35	28	27	27
Number of animals with multiple masses	1	2	0	2	15	11	13	9
Mean number of masses per animal	1.1 ± 0.38	1.2 ± 0.66	1.0 ± 0.00	1.1 ± 0.36	1.7 ± 0.87	1.7 ± 0.98	2.0 ± 1.26	1.5 ± 0.85
Mean number of days to first mass	645 ± 82	571 ± 148	552 ± 137	585 ± 160	509 ± 122	489 ± 100	480 ± 107	497 ± 152

None significantly different from control group using Fisher's Exact Test.

Administered doses – Maximum tolerated dose

Administered doses were 250, 1000, and 5000/3000 ppm (males/females), equating to consumed levels of 10, 41, and 217 mg/kg bw/day respectively for males and 13, 53, and 167 mg/kg bw/day respectively for females. The doses for this study were 2-3 times lower than the ones administered for the 90-day repeated-dose toxicity study; therefore, HSE has provided additional consideration regarding the adequacy of the administered dose range. There were statistically significant reductions in body weight and body weight gains in both sexes at the top-dose, accompanied with clinical chemistry findings, organ weight changes and histopathology findings (please refer to findings below).

Thus, it is concluded that for both sexes, the top-dose complies with the recommendations laid out in the OECD guideline Nº 453 regarding the maximum tolerated dose (paragraph 25), in that '*the highest dose level should be chosen to identify the principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death. The highest dose level should be normally chosen to elicit evidence of toxicity, as evidenced by, for example, depression of body weight gain (approximately 10%)*.'

Therefore, the study is adequate to investigate the carcinogenicity potential of bixlozone in rats, with the MTD reached in both sexes at the top-dose of 217 mg/kg bw/day for males and 167 mg/kg/bw/day for females.

Body weights, body weight gains & food consumption

Test substance-related lower mean body weights were noted in the top-dose group males (6-11%) and females (7-18%) throughout the study, generally reaching statistical significance when compared to the control groups. Body weights changes correlated well with lower mean cumulative body weight gains (generally reaching statistical significance compared to the control groups; 9-14% in males and 14-24.5% in females). In general, the effects seen for the top-dose females on mean body weights correlated with effects on food consumption, especially during the chronic toxicity phase (0-52 weeks).

At Day 49 (week 7), the dose administered to the 4000 ppm female group was reduced to 3000 ppm based on severe adverse effects, characterised by a mean body weight loss approaching 10 %, corresponding lower mean body weight gains of 18-20 % and lower mean food consumption (9-12 %) noted during the first 6 weeks of the study period. Consequently, there was an improvement in the condition of these animals by week 13: the severity of the effects observed at week 13 was reduced compared to week 7, with no reductions in body weight and a higher mean body weight gain and mean food consumption compared to week 7 (Table B.6.5.5.5).

By the end of the study the body weight and body weight gains reductions for the 3000 ppm female group exceeded the 10 % changes recommended in paragraph 25 of the OECD Guideline Nº 453 (mean body weights

and body weight gain was 14 % and 22 % lower compared to controls). However, since the mortality rates were similar across all female groups including controls and that no statistical difference in the survival between animal groups of same sex was noted, HSE concludes that the level of toxicity noted for this dose group did not compromise the validity of the study and that the reduction of the dose had possibly prevented excessive toxicity occurring at this dose.

Overall statistically significant adverse lower mean body weights and body weight gains were observed at the top-dose (5000 ppm in males and 3000 ppm in females) in both sexes throughout the study.

Table B 6.5.1.5: Body weight development and food consumption in the two-year rat study with bixlozone

	Males				Females			
Dose (ppm)	**0**	**250**	**1000**	**5000**	**0**	**250**	**1000**	**4000/3000[a]**
Dose (mg/kg bw/day)	**0**	**10**	**41**	**217**	**0**	**13**	**53**	**167**
	Body weight (g)							
Week 7	461 ± 37.5	459 ± 33	463 ± 39	**434 ±39****	254 ± 19	254 ± 23	254 ± 20	**234 ± 18****
% from control		-0.4	0.4	**-6**		0	0	**-8**
Week 13	539 ± 45	539 ± 45	539 ± 51	**505 ±48****	282 ± 23	284 ± 26	283 ± 24	**262 ± 21****
% from control		0	0	**-6**		0.7	0.4	-7
Week 52	752 ± 94	744 ± 74	745 ± 87	**700 ±80****	407 ± 52	402 ± 62	402 ± 51	**348 ± 40****
% from control	-	-1.1	-0.9	-7	-	-1.2	-1.2	**-14.5**
Week 80	828 ± 106	802 ± 94	798 ± 107	**756 ±110****	476 ± 86	454 ± 82	474 ± 81	**392 ± 76****
% from control		-3	-4	**-9**		-5	-0.4	**-18**
Week 104	793 ± 112	781 ± 99	781 ± 141	709 ± 90	520 ± 58	468 ± 79	496 ± 66.5	446 ± 77
% from control		-1.5	-1.5	-11		-10	-5	-14
	Body weight gain (g)							
Week 0-7	248± 29	248 ± 30	251 ± 33	**224 ± 30****	108 ± 14	107 ± 20	108 ± 15	**89 ± 13****
% from control		0	+1.2	**-10**		-1	0	**-18**
Week 0-13	326± 38	327 ± 41	327 ± 45	**294 ± 39****	136 ± 19	137 ± 23	137 ± 19	**117 ± 17****
% from control		+0.3	+0.3	**-10**		+0.7	+0.7	**-14**
Week 0-52	539 ± 90	532 ± 70	532 ± 80	**489 ± 72****	261 ± 48	255 ± 57.5	257 ± 48	**203 ± 39****
% from control		-1.3	-1.3	**-9**		-2	-1.5	**-22**
Week 0-80	614 ± 101	591 ± 92	588 ± 102.5	**545 ± 101****	330 ± 84	310 ± 76	327 ± 79	**249 ± 74****
% from control		-4	-4	**-11**		-6	-0.9	**-24.5**
Week 0-104	579 ± 113.5	569 ± 94.5	574 ± 138	497 ± 87	373 ± 53	325 ± 78	349 ± 68	302 ± 75
% from control		-1.7	-0.9	-14		-13	-6	-19

* P<0.05, **P<0.01 determined as statistically significantly different to control using appropriate statistical test
N Number of animals tested
[a] = For females, dosage level was lowered on Study Day 49 from 4000 ppm to 3000 ppm due to excessive toxicity.

Toxicokinetics (Table B 6.1.1.6)

Consistent with the previous toxicokinetics findings from the 28-day repeated dose study (Sections **Error! Reference source not found.** & B.6.1.1.3), plasma concentrations of bixlozone increased with dose up to the maximum dose in both sexes and females were more systemically exposed to bixlozone than males. The highest exposure was recorded at day 1 for both sexes. On the following sampling days, plasma concentrations were noted to be lower compared to day 1 from 1000 ppm in both sexes, and concentrations remained stable up to the end of the study. Test substance-related lower mean food consumption, which could partly explain the lower plasma concentrations of bixlozone observed after study initiation, was noted in the 3000 ppm females and the 5000 ppm males compared to controls, especially during the chronic toxicity phase (0-52 weeks). Since bixlozone exposure remained higher in females than in males despite females consuming less food than males (Table B 6.5.1.5), it is possible that males exerted an enhanced metabolism of the test substance over time compared to females.

Consistent with the findings from the short-term 28-day rat study (Section **Error! Reference source not found.**), there is no indication that bixlozone accumulates upon chronic dosing.

Clinical pathology (Table B 6.5.1.6)

There was no test substance-related or biologically relevant effect on haematological parameters; however, some treatment-related (and statistically significant) alterations in serum chemistry were noted in females at the top-dose.

As observed in the short-term repeated-dose toxicity studies in the rat (Section B.6.3), there were changes in metabolism parameters with an increase in serum cholesterol in top dose females at week 26 and 52 (+53 and +50.5 % compared to controls, respectively). These changes are considered to be treatment-related and adverse since they were already observed in the 90-day study in the rat (Section **Error! Reference source not found.**).

With regard to serum protein parameters, there was a statistically significant increase in total protein and albumin in females at the top-dose at week 52 (+8 and +7 % respectively). The increase in albumin, the main major serum binding protein for calcium, was associated with an increase in calcium. These findings were also observed in repeated-dose toxicity studies and are considered treatment-related and adverse.

Higher mean gamma glutamyl transferase (GGT) values were noted in the 5000 ppm males (+500 %) at study week 26, and in the 1000 and 5000 ppm males at study week 52 (+1200 and +700 %, respectively); a change in this parameter may be indicative of liver toxicity. However, HSE notes that individual values within each group were highly variable, and since these changes were not accompanied with increased serum bilirubin and were not observed in females, their validity and biological relevance are considered questionable.

Urinalysis revealed no unusual findings.

Overall treatment-related increases in serum chemistry parameters indicative of adverse effects in the liver (serum cholesterol, albumin, calcium, total protein), were observed in the top-dose females but not in males during the chronic toxicity phase; these effects correlated with the other effects seen on the liver in these animals (organ weight changes and histopathology described further below). The findings are also consistent with the toxicokinetics data indicating that females are more systemically exposed to bixlozone than the males.

Table B 6.5.1.6: Summary of serum chemistry findings in the two-year rat study with bixlozone

	Males				Females			
Dosage (ppm)	**0**	**250**	**1000**	**5000**	**0**	**250**	**1000**	**4000/3000**
(mg/kg bw/day)	**0**	**10**	**41**	**217**	**0**	**13**	**53**	**167**
Clinical chemistry (Week 26 and Week 52 –end of chronic toxicity period)								
Cholesterol (mg/dL)								
Week 26	81 ± 17.4	79 ± 13.8	85 ± 37.3	91 ± 14.4	78 ± 22.2	88 ± 19.5	79 ± 23.8	**119 ± 18.2****
% from control						+13	+1.3	**+53**
Week 52	98 ± 18.0	94 ± 22.9	98 ± 30.6	97 ± 14.8	99 ± 21.4	95 ± 29.8	105 ± 18.4	**149 ± 50.5****
% from control						-4	+6	**+50.5**
Calcium (mg/dL)								
Week 26	10.5 ± 0.31	10.6 ± 0.20	10.5 ± 0.28	10.6 ± 0.24	10.5 ± 0.18	10.5 ± 0.31	10.8 ± 0.27	**10.9 ± 0.22****
% from control						0	+3	**+4**
Week 52	10.6 ± 0.16	10.6 ± 0.30	10.5 ± 0.23	10.5 ± 0.18	10.5 ± 0.18	10.5 ± 0.31	10.8 ± 0.27	**10.9 ± 0.22****
% from control						0	0	**+4**
Albumin (g/dL)								
Week 26	4.0 ± 0.10	4.1 ± 0.16	4.0 ± 0.15	4.14 ± 0.18	4.6 ± 0.30	4.8 ± 0.31	4.7 ± 0.32	4.9 ± 0.22
% from control						+4	+2	+6.5
Week 52	4.0 ± 0.14	4.0 ± 0.2	4.0 ± 0.18	4.2 0.14	4.5 ± 0.18	4.4 ± 0.35	4.6 ± 0.26	**4.8 ± 0.22***
% from control						-2	+2	+7
Total protein (g/dL)								
Week 26	6.9 ± 0.24	7.1 ± 0.35	7.0 ± 0.38	7.0 ± 0.40	7.7 ± 0.49	8.0 ± 0.65	7.9 ± 0.40	8.2 ± 0.43
% from control						+4	+3	+6.5
Week 52	6.9 ± 0.24	5.7 ± 0.3	6.8 ± 0.24	7.1 ± 0.25	7.6 ± 0.32	7.4 ± 0.55	7.7 ± 0.25	**8.2 ± 0.45***
% from control						-3	+1.3	**+8**
GGT (U/L)								
Week 26	0.1 ± 0.32	0.2 ± 0.63	0.2 ± 0.63	0.6 ± 1.07	0.1 ± 0.32	0.0 ± 0	0.0 ± 0	0.0 ± 0
% from control		+100	+100	+500				
Week 52	0.1 ± 0.32	0.0 ± 0	1.3 ± 3.47	0.8 ± 0.79	0.1 ± 0.32	0.0 ± 0	0.0 ± 0	0.0 ± 0
% from control		-100	+1200	+700				

* P<0.05, **P<0.01 determined as statistically significantly different to control using appropriate statistical test

Organ weight changes (Table B 6.5.1.7)

Consistent with repeated-dose toxicity studies in the rat, statistically significant changes in liver weights were seen at week 52 (chronic toxicity groups) and comprised increased liver weights and relative liver weight to body weights in top-dose females (+17 and +34 % compared to controls, respectively) and increased relative liver weights to body weights in males (+19 % compared to control males). Since these weight changes are > 15 % and are accompanied with serum chemistry alterations (increased cholesterol, total protein and albumin) and histopathology findings of hepatocellular hypertrophy and vacuolation (please refer to histopathology section further below), they are considered treatment-related and adverse at top-dose for both sexes.

By the end of the study (week 104, carcinogenicity groups), absolute and relative liver weights were only > 15 % for the top-dose males compared to controls (+ 20 % absolute and + 34 % relative), and they were also considered treatment-related and adverse for the same reasons as above.

In contrast with the 90-day rat study, there were no adverse effects noted at weeks 52 and 104 for the kidneys in both sexes up to the top-dose (Section **Error! Reference source not found.**). Statistically significantly higher thyroid/parathyroid weights (absolute and relative to body weights) were noted at week 52 in males for the 1000 ppm (34 and 17 % respectively, the latter being not statistically significant) and 5000 ppm (23 and 17 % respectively) group compared to the control group; at week 104 the increase was noted in the top-dose males

without statistical significance. However, since there was not a clear dose -related response at both time points, and no associated microscopic findings, these organ weight changes in males were not considered adverse.

There were no other biologically relevant or statistically significant effects on the weights of any other organ.

Overall treatment-related and adverse increases in liver weight were observed at week 52 in males and females at top-dose and at week 104 in males only.

Table B 6.5.1.7: Selected organ weights from the combined chronic toxicity / carcinogenicity rat study with bixlozone

	Males				Females			
Dosage (ppm)	**0**	**250**	**1000**	**5000**	**0**	**250**	**1000**	**4000/3000**
mg/kg bw/day	**0**	**10**	**41**	**217**	**0**	**13**	**53**	**167**
	Organ weights (g) at week 52							
Final body weight	692 ± 67.9	703 ± 74.5	731 ± 62.6	654 ± 77.2	375 ± 37.2	370 ± 59.8	366 ± 48.5	326 ± 23.7
Liver weight	18.57 ± 1.98	18.47 ±1.68	19.27 ± 1.81	20.83 ± 2.74	10.45 ± 1.44	10.96 ± 1.35	10.93 ± 1.60	**12.22 ± 1.21***
% difference from controls		-0.5	+4	+12		+5	**+5**	**+17**
Relative to body weight	2.69 ± 0.163	2.64 ± 0.150	2.64 ± 0.114	**3.19 ± 0.26****	2.81 ± 0.47	2.99 ± 0.31	3.00 ± 0.41	**3.77 ± 0.53****
% Relative weight		-2	-2	**+19**		+6	+6	**+34**
Thyroids weight	0.038 ± 0.007	0.045 ± 0.006	**0.051 ± 0.01****	0.047 ± 0.008	0.038 ± 0.006	0.036 ± 0.008	0.042 ± 0.01	0.039 ± 0.007
% difference from controls		+18	**+34**	+23		-5	+10	+3
Relative to body weight	0.006 ± 0.001	0.007 ± 0.008	**0.007 ± 0.001***	**0.007 ± 0.001****	0.01 ± 0.002	0.01 ± 0.001	0.01 ± 0.004	0.01 ± 0.002
% Relative weight		+17	**+17**	**+17**		0	0	0
	Organ weights (g) at week 104							
Final body weight	763 ± 108	749 ± 98.2	754 ± 141.3	675 ± 90.2	491 ± 56.5	441 ± 78.7	468 ± 67.6	419 ± 75.0
Liver weight	18.33 ± 2.63	19.92 ± 2.49	18.63 ± 3.00	**21.97 ± 3.40****	14.7 ± 2.8	12.7 ± 2.14	14.37 ± 2.12	13.6 ± 2.0
% difference from controls		+9	+1.6	**+20**		-14	-2	-7
Relative to body weight	2.41 ± 0.17	**2.69 ± 0.40****	2.498 ± 0.32	**3.26 ± 0.31****	3.03 ± 0.68	2.92 ± 0.47	3.11 ± 0.51	3.29 ± 0.44
% Relative weight		**+12**	+4	**+35**		-4	+3	+9
Thyroids weight	0.035 ± 0.012	0.035 ± 0.01	0.035 ± 0.01	0.04 ± 0.01	0.031 ± 0.02	0.032 ± 0.03	0.051 ± 0.08	0.032 ± 0.02
% difference from controls		0	0	+14		+3	+65	+3
Relative to body weight	0.005 ± 0.002	0.005 ± 0.001	0.005 ± 0.001	0.006 ± 0.002	0.007 ± 0.005	0.007 ± 0.007	0.014 ± 0.03	0.008 ± 0.006
% Relative weight		0	0	+20		0	+100	+14

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test

Ophthalmic examinations

There were no test substance-related effects observed during the ophthalmic examinations.

Macroscopic examinations (Table B 6.5.1.8)

In males a higher incidence of distended urinary bladder was observed in the unscheduled deaths of the 1000 and 5000 ppm groups. This finding was not associated with microscopic changes or test substance-related alterations in urinalysis parameters. No other relevant finding was noted in the unscheduled deaths or scheduled necropsy groups. Therefore, this finding was not considered adverse.

Table B 6.5.1.8: Summary of macroscopic findings in combined chronic toxicity / carcinogenicity rat study with bixlozone

Macroscopic findings - found dead or euthanized moribund or in extremis								
	Males				Females			
Dosage (ppm)	**0**	**250**	**1000**	**5000**	**0**	**250**	**1000**	**4000/3000**
(mg/kg bw/day)	**0**	**10**	**41**	**217**	**0**	**13**	**53**	**167**
Number of animals	**35**	**27**	**29**	**27**	**37**	**33**	**37**	**38**
Urinary bladder, distended	1	0	5	5	1	0	0	1
Urinary bladder, dark red contents	0	0	1	2	None	None	None	None

Histopathology -Non-neoplastic findings (Table B 6.5.1.10)

At week 52 treatment-related and dose-dependent increased incidence in hepatocellular hypertrophy was noted in males from 250 ppm and in females from 1000 ppm. The hypertrophy was characterised by enlarged centrilobular hepatocytes, and the severity increased with the dose. Minimal to mild centrilobular hepatocellular vacuolation was noted in all male groups included controls (but not in females), and the incidence increased with the dose from 1000 ppm.

At week 104 the findings were similar to the ones observed at week 52 for both sexes, with hypertrophy increasing with dose in both males and females from 1000 ppm. The incidence of minimal to mild centrilobular hepatocellular vacuolation was increased only in top dose males and no clear dose-response was seen in females.

Thus, the liver microscopic findings correlated well with the other relevant liver effects highlighted in this study (increased absolute and relative liver weights, increased cholesterol levels) and with the findings in the preceding short-term repeated-dose toxicity studies in the rat.

In contrast with the 90-day repeated-dose study, there were no histopathology findings in the thyroid; no other treatment-related microscopic findings in any other organ were observed.

Lastly, a marginal, but statistically significant increase in uterine glandular polyps (4/60 vs 1/60 in controls) was seen at week 104 in top-dose females. Following a request for further information to consider further the biological significance of the finding the applicant consulted a Pathology Working Group to review the finding according to current published nomenclature and diagnostic criteria, without knowledge of animal identification, group designation, or previous diagnosis. HSE reviewed the report and a summary of the results is presented after the Table B 6.5.1.9.

Overall treatment-related and dose-dependent increased incidence in hepatocellular hypertrophy was noted in males from 250 ppm and in females from 1000 ppm, and hepatocellular vacuolation was increased in males from 1000 ppm. These changes were considered adverse at top-dose only since they correlated with adverse liver weight and serum chemistry changes.

Table B 6.5.1.10: Selected non-neoplastic microscopic findings from the combined chronic / carcinogenicity rat study with bixlozone

	Males				Females			
Dosage (ppm)	**0**	**250**	**1000**	**5000**	**0**	**250**	**1000**	**4000/3000**
(mg/kg bw/day)	**0**	**10**	**41**	**217**	**0**	**13**	**53**	**167**
Microscopic findings at Week 52								
Liver (N = 10)								
Hypertrophy, Hepatocellular	0	1	3	7	0	0	8	10
Minimal	-	1	3	4	-	-	6	6
Mild	-	0	0	3	-	-	2	4
Vacuolation, hepatocellular	2	2	6	8	0	1	1	0
Minimal	1	2	4	5	-	-	-	-
Mild	1	-	2	3	-	1	1	-
Hepatocyte, multinucleated, mild	0	0	0	0	0	0	1	1
Microscopic findings at Week 104								
Liver								
Number of tissues examined	45	47	49	47	47	48	48	46
Hypertrophy, hepatocellular (% incidence)	0	0	5 (10)	37 (79)	0	0	2 (4)	34 (74)
Minimal	-	-	5	24	-	-	2	22
Mild	-	-	-	13	-	-	-	12
Vacuolation, hepatocellular (% incidence)	6 (13)	7 (15)	3 (6)	15 (32)	6 (13)	4 (8)	14 (29)	6 (13)
Minimal	2	4	3	13	3	3	10	6
Mild	1	1	-	2	3	1	2	-
Moderate	2	2	-	-	-	-	2	-
Severe	1	-	-	-	-	-	-	-
Vacuolation, macro vesicular	0	0	0	1	0	0	0	0
Minimal	-	-	-	1	-	-	-	-
Uterus								
N examined					60	50	49	60
Glandular polyp					1	1	0	4
1-sided pairwise comparison (p)[a]						*0.814*	*1.000*	*0.1459*
1-sided trend test (p)[b]								*0.0478*

ND

[a] 1-sided pairwise comparison of 0 ppm with active treatment group
[b] 1-sided trend test including 0 ppm and active treatment groups
Statistical Significance: Rare tumour - p<0.025 (trend), p<0.05 (pairwise); Common tumour - p<0.005 (trend), p<0.01 (pairwise)
* Statistically significant at the defined significance level
ND = Not determined

Additional information provided by the applicant on the incidence in uterine polyps

Following a request for further information to consider further the biological significance of the neoplastic and non-neoplastic findings from this study the applicant submitted a Pathology Working Group (PWG) Report (Pathology Working Group Review Of Selected Neoplasms From Rats In An 2-Year Oral (Dietary) Combined Chronic Toxicity And Carcinogenicity Study With Toxicokinetic Measurements Of F9600 Technical. Thomas, H. C. 2020 – Report FMC-53830). The PWG reviewed the finding according to current published nomenclature and diagnostic criteria, without knowledge of animal identification, group designation, or previous diagnosis. HSE reviewed the report and considered the case acceptable; a summary of the results is presented below.

The PWG panel based their diagnosis of cervical and uterine polyps on the INHAND document on proliferative and non-proliferative lesions in the female reproductive system in rodents (Dixon D., *et al.* (2014) Non-proliferative and Proliferative Lesions of the Rat and Mouse Female Reproductive System. J Toxicol Pathol, 27(3-4):1S-107S).

Polyps may arise from the vagina, uterus or cervix and typically protrude as a mass into the lumens of all or any of these structures. In rats, two basic types of polyps are generally recognised in the female reproductive tract and include glandular polyps and endometrial stromal polyps, but neither, by definition is typically invasive. The PWG panel criteria for endometrial stromal polyps included having less than 50 % of the polyp composed of entrapped glands. Thus all uterine glandular polyps (including the 4/60 at the top dose) were reclassified as uterine endometrial stromal polyps. It is noted that uterine stromal polyps are present at higher incidence (8/60) in controls compared to all treated groups.

Thus, following the re-classification of the uterine glandular polyps by the PWG, an increase in uterine polyps following treatment with bixlozone is no longer apparent. A comparison of the incidence of uterine polyps, as determined by the study pathologist and the PWG, are summarised in the table below:

Comparison of the Study Pathologist and Pathology Working Group Diagnosis for Uterine Polyps in Female Rats

Group	*1*		*2*		*3*		*4*	
Dose (ppm)	*0*		*250*		*1000*		*3000*	
	SP	***PWG***	***SP***	***PWG***	***SP***	***PWG***	***SP***	***PWG***
Uterus (No. Examined)	*60*	*8*	*52*	*4*	*50*	*1*	*60*	*5*
Endometrial Glandular Polyp	*1*	*0*	*1*	*0*	*0*	*0*	*4*	*0*
% Incidence	*1.67*	*0.00*	*1.92*	*0.00*	*0.00*	*0.00*	*6.67*	*0.00*
Endometrial Stromal Polyp	*7*	*8*	*3*	*4*	*1*	*0[a]*	*1*	*4[b]*
% Incidence	*11.67*	*13.33*	*5.77*	*7.69*	*2.00*	*0.00*	*1.67*	*6.67*

SP – Study Pathologist
PWG – Pathology Working Group
a This animal was diagnosed by PWG as a stromal sarcoma within the polyp.
b One animal was diagnosed by PWG as within normal limits.

Histopathology -Neoplastic findings (Table B 6.5.1.11)

Following a 2-year administration of bixlozone to rats, there were no increases (relative to controls) in the tumour incidence in any of the main target organs identified in the repeated-dose studies (liver, kidneys) in both sexes; any incidences seen did not show a clear dose-response, were small in magnitude or were also seen in controls. Increased incidences of tumours were only seen for the skin in males and the thyroid in females.

Skin tumours in males

Regarding males, there was a non-statistically significant increase in the incidence of skin fibrosarcoma (malignant tumours; 3/60-5% vs 0/60 in controls) and fibroma (1/60-1.7% vs 0/60 in controls) at the top-dose of 5000 ppm in comparison to the control, with 4 males bearing fibrosarcoma or fibroma in the skin. This is consistent with the increase in the number of top-dose males with palpable masses at the external examination. Fibrosarcoma was considered the cause of death for 2 of these animals (one euthanised *in extremis* on study day 234, and one that died on study day 708). The incidence of fibroma is even below the mean incidence of the laboratory HCD provided, whilst for fibrosarcoma, the incidence is above the mean incidence value but well within the HCD range. There were no neoplastic findings noted at the lower doses.

Although the HCD provided is large (derived from 31 male control groups in 21 studies conducted from February 2001 to July 2013), the data are not fully compliant with the data requirements laid out in Reg 283/2013 (section 5, point 3) which specify that HCD should "cover a five-year period, centred as closely as possible on the date of the index study". Nevertheless, these extended HCD, which can be taken into account in a WoE approach, suggest that skin fibromas are relatively common tumours since the HCD range (1.43 – 8.33 %) shows that at least 1 control animal in every study (included in the HCD) is found with such tumours. Regarding fibrosarcomas it is also noted that 1 concurrent control female showed a fibrosarcoma (with none in the treated female groups); since the HCD range for this finding is 1.43 % - 7.14 % in males (with at least 1

control animal in every study found with such tumours) it suggests that the incidence in the concurrent control male group in this study (0/60) was unusually low.

Considering the biological plausibility of the finding, it is noted that the skin has not been identified as a target organ of toxicity of bixlozone in any other repeated-dose toxicity studies (Section B.6.3) and bixlozone was found not to be acutely toxic via the dermal route or a skin irritant (Section B.6.2). Moreover, a tissue distribution study showed that bixlozone and/or its metabolites were only found at low levels in the skin after administration of an oral dose of 500 mg/kg bw (Section B.6.1.1.4) which is much higher than the highest dose tested in males in this study (217 mg/kg bw/day).

Moreover, the finding was sex specific, with an increased incidence observed in males only. However, the available kinetics data contrast with this sex specificity in response since in rats the females show a higher systemic exposure than males (Table B 6.1.1.6) and yet females do not present any tumours up to the top dose (4000 / 3000 ppm). Lastly, since no skin tumours were observed in the mouse (Section B.6.5.2), the skin tumours seen in this study are also species specific.

The applicant submitted a review of the incidence of these skin tumours by a PWG panel. The PWG confirmed the incidences determined by the study pathologist. Therefore, these data have not been considered further by HSE and they have not been presented here.

Overall, considering the sex and species specificity of the response, the low biological plausibility of the finding, the inconsistency between the sex-specificity of the response and the higher systemic exposure in females, and the fact that the incidence was clearly within the range of the extended (2001-2013) HCD supplied, HSE concludes that these skin tumours in male rats at the top dose are chance findings unrelated to treatment.

Thyroid tumours in females

Regarding females, there was a non-statistically significant but dose-related increase in the incidence of follicular cell adenomas (benign tumours; 2/60 (3.3 % incidence) vs 0/60 in controls) and of the follicular cell carcinoma (1/60 (1.7 % incidence) vs 0/60 in controls) in the thyroid gland at the top-dose of 3000 ppm in comparison to controls.

The HCD provided for these tumour findings are not fully compliant with the data requirements laid out in Reg 283/2013 (section 5, point 3) because they cover more than 5 years, however they are from the same laboratory and strain of rat and derived from a reasonable number of studies; thus, they can be considered by HSE in a WoE approach. These significantly extended HCD (date range 1999 – 2017) show that at the top dose both tumour incidences are slightly higher than the mean % incidence but well within the HCD range (Table B 6.4.1.3.10). The applicant also provided the maximum control numerical and % incidence values derived from studies conducted between 2009 and 2017 i.e. performed around the date (2014) of the current study: the maximum numerical incidence was 3 (4.7 % incidence) for follicular cell adenoma and 1 (1.7 %) for follicular cell carcinoma. Thus the thyroid tumour incidences seen in the study in females at the top dose are consistent with the top of the range of the control groups monitored around the time of the study.

The finding is sex specific since no relevant or dose-related increase in incidence was observed in males. Moreover, it is noted that the incidence of follicular cell adenoma (benign) in the control male group is similar (3.4 %) to the incidence seen in the top dose female group (3.3 %) and that the incidence of follicular cell carcinoma was for both sexes very low (maximum of 1 case per 50 / 60 rats per group) and clearly not dose-related.

The available kinetics data appear to support the sex specificity of the response since in rats the females show a higher systemic exposure than males (Table B 6.1.1.6). However, a tissue distribution study showed that less than 0.1 % of the AD was found in the thyroid in both sexes following oral administration of bixlozone (Table B 6.1.1.12) with the thyroid of males being more exposed than females' following a single high oral dose (500 mg/kg bw) or a repeated low oral dose (5 mg/kg bw/day, 14 days).

Considering the biological plausibility of the finding, it is noted that there were no other associated findings noted in the thyroid (e.g. hyperplasia, hypertrophy) in the study to support the tumorigenic response, even though the thyroid was identified as a target organ of toxicity in the 90-day rat study (Section **Error! Reference source not found.**). Yet, the histopathology changes in the 90-day study were seen at doses higher than the top dose tested in this carcinogenicity study, with mild follicular cell hypertrophy observed in both sexes at the top dose of 505 / 351 mg/kg bw/day only and without associated changes in the thyroid weights (Section **Error! Reference source not found.**). There were no histopathology findings seen at the lower doses of 121 / 150 mg/kg bw/day. Therefore, the biological plausibility of these thyroid tumour findings appears to be low.

The applicant submitted a review of the incidence of these thyroid tumours by a PWG panel. The PWG confirmed the incidences determined by the study pathologist. Therefore, these data have not been considered further by HSE and they have not been presented here.

Overall, considering the sex specificity of the response, the low incidence of the tumours and the low biological plausibility of the finding, HSE conclude that the thyroid tumours observed in female rats at the top dose are chance findings unrelated to treatment.

Overall, there were no treatment-related neoplastic findings identified in this study in the rat.

Table B 6.5.1.11: Selected neoplastic microscopic findings in the chronic/carcinogenicity rat study with bixlozone

Dose-levels (ppm)	Males				HCD Mean % incidence (incidence range)	Females				HCD Mean % incidence (incidence range)
	0	250	1000	5000		0	250	1000	3000	
Dose (mg/kg bw/day)	**0**	**10**	**41**	**217**		**0**	**13**	**53**	**167**	
Skin *N examined*	60	50	50	60		*60*	*50*	*50*	*60*	
Fibroma, benign (% incidence)	0	0	0	1 (1.67)	2.3[c] (1.43-8.33)	0	0	0	0	
Fibrosarcoma, malignant (% incidence)	0	0	0	3 (5.0)	1.26[c] (1.43 - 7.14)	1	0	0	0	
1-sided pairwise comparison (p)[a]		*1.000*	*1.000*	*0.1575*		*ND*				
1-sided trend test (p)[b]	*0.0206*									
Thyroid gland *N examined*	*58*	*50*	*47*	*60*		*60*	*50*	*49*	*60*	
C cell adenoma, benign	7	10	4	5		5	5	6	5	
C cell carcinoma, malignant	1	0	0	0		2	1	0	1	
Combined C-cell carcinoma/adenoma	8	10	4	5		7	6	6	6	
Follicular cell adenoma, benign (% incidence)	2 (3.44)	0 (0.00)	3 (6.4)	2 (3.33)		0 (0.00)	0 (0.00)	1 (2.04)	2 (3.33)	HCD 1999-2017 1.30[d] (0.0 – 6.12) HCD 2009-2017 (1.54 – 4.69)
1-sided pairwise comparison (p)[a]	*ND*						*1.000*	*0.480*	*0.220*	
1-sided trend test (p)[b]						*0.0408*				
Follicular cell carcinoma, malignant	0	0	1	0		0	1	0	1 (1.7)	HCD 1999-2017 0.41[d] (0.0 – 3.33) HCD 2009-2017 (1.43 – 1.67)
1-sided pairwise comparison (p)[a]	*ND*						*0.567*	*0.480*	*0.124*	
1-sided trend test (p)[b]						*0.0255*				

[a] 1-sided pairwise comparison of 0 ppm with active treatment group
[b] 1-sided trend test including 0 ppm and active treatment groups
Statistical Significance: Rare tumour - p<0.025 (trend), p<0.05 (pairwise); Common tumour - p<0.005 (trend), p<0.01 (pairwise)
* Statistically significant at the defined significance level
ND = Not determined
NA = not available
[c] HCD mean and range for Crl:CD(SD) male rats – sub-chronic ([redacted] laboratory): 08 Feb 2001 - 08 Jan 2013; Number of Studies/Control Groups for males: 21 / 31;
[d] HCD mean and range for Crl:CD(SD) female rats – sub-chronic ([redacted] laboratory): June 1999 - Jan 2017; Number of Studies/Control Groups for females: 32 / 48

Conclusion

In a GLP- and OECD-compliant combined chronic toxicity / carcinogenicity study, bixlozone technical was administered in the diet of Crl:CD (SD) rats for 52 and 104 consecutive weeks at doses of 250, 1000, and 5000/3000 ppm (males/females), equating to consumed levels of 10, 41, and 217 mg/kg bw/day respectively for males and 13, 53, and 167 mg/kg bw/day respectively for females.

The overall percent survival at week 104 was low in this study (below the 50 % recommended in the OECD Guideline 116 on the conduct and design of chronic toxicity and carcinogenicity studies). However, since the survival rates at 18 months were well above 50 % and those at 21 months were around 50 % the UK considers that overall the power of the study was not compromised.

Systemic toxicity was observed at the top-dose in both sexes throughout the study (5000 ppm 3000 ppm in males / females, equivalent to 217 and 167 mg/kg bw/day respectively) which indicated that the top-dose was sufficiently high in these animals; body weight and body weight gain were statistically significantly reduced compared to the control groups. For females, the MTD was possibly slightly exceeded since the reductions in body weight and body weight gains were greater than 10 %; however, since the survival rate for this group was similar to the lower dose groups, the finding is not considered to undermine the validity of the study.

Consistent with the findings of the short-term repeated-dose studies, the liver was identified as a target organ. Treatment-related increases in parameters indicative of adverse effects in the liver (serum cholesterol, albumin, calcium, total protein), were observed in the top-dose females and correlated with the liver weight changes and associated hepatocellular hypertrophy findings observed in these animals. In males, treatment-related effects in the liver were observed from 1000 ppm (and considered adverse at 5000 ppm) and were characterised by liver weight changes at 5000 ppm and hepatocellular hypertrophy findings.

Overall, the NOAEL for systemic chronic toxicity in the rat is set at 1000 ppm (41 / 53 mg/kg bw/day in males / females respectively) with a LOAEL set at 5000/3000 ppm (217 / 167 mg/kg bw/day in males / females respectively) based on adverse effects observed in the liver at this dose (serum chemistry changes, liver weight changes and histopathology findings), effects on body weights in both sexes and uterine glandular polyps in females.

Regarding neoplastic findings, there were no treatment-related findings identified for both sexes up to the top dose tested in this study. Therefore, the NOAEL for carcinogenicity in the rat is set at the top dose of 5000 / 3000 ppm (167 / 217 mg/kg bw/day in males and females, respectively).

[redacted] (2017)

Table B 6.5.1.12: Summary of the 2-year combined chronic toxicity / carcinogenicity study in the rat

Method, Species, test substance *Acceptability*	Doses	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
Dietary 24-month **Rat** (Crl:CD (SD) rats, males and females) GLP OECD 453 (2009) Deviations: none ([redacted] (2017)) ***Acceptable***	F9600 technical Batch PL14-0049 Purity 96 % 0, 250, 1000, 5000/3000 ppm Equivalent to: Males: 0, 10, 41, 217 mg/kg bw/day Females: 0, 13, 53, 167 mg/kg bw/day	*Carcinogenicity* 217 / 167 (5000 / 3000 ppm in M/F)	*Carcinogenicity* > 217 / 167 (5000 / 3000 ppm in M/F)	**Chronic phase – 12 months** 5000/3000 ppm (217 / 167 mg/kg bw/d) ↓ BW-gain in females (10%** days 1-344) ↑ liver weight in F (18%*absolute & 11%* relative) 1000 ppm & 250 ppm No adverse effects **Carcinogenicity phase – 24 months** *Non-neoplastic findings* 5000/3000 ppm (217 / 167 mg/kg bw/d) Dermal atonia and thin body condition in F ↓ body weight gain for both sexes (9-14%** M and 14-24.5%** F) *Organ weights* ↑ relative liver weight > 15 %** (both sexes) ↓ cholesterol**, albumin*, calcium**, total protein* (F) *Histopathology findings - liver* Hepatocellular hypertrophy: 7/10 at 52 weeks & 79 % incidence at 104 weeks (M) 10/10 at 52 weeks & 74 % incidence at 104 weeks (F) Hepatocellular vacuolation: 7/10 at 52 weeks & 32 % incidence at 104 weeks (M) 10/10 at 52 weeks & 74 % incidence at 104 weeks (F) 1000 & 250 ppm No adverse effects *Neoplastic findings* No treatment-related findings.
		Systemic chronic toxicity 41 (males) 53 (females) (1000 ppm)	*Systemic chronic toxicity* 217 (males) 167 (females) (3000 ppm)	

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test

[redacted] (2017)

B.6.5.2. 18-months carcinogenicity study in mice

An 18-month oral carcinogenicity study conducted in CD-1 mice is available.

Toxicokinetic parameters have been included in this study; short summaries of the toxicokinetic findings are presented in this Section but for further details please refer to Section 6.1. of the DAR. Analyses were conducted using a validated high performance liquid chromatography method using UHPLC-MS/MS detection. The method validation is within a separate study (Lucarell, 2016b; WIL Research, USA; Study No. WIL-105141) and the data are presented in Volume 3, Section CA B.4, Point CA 4.1.2(c).

Study	An 18-month Oral (Dietary) Carcinogenicity Study with Toxicokinetic Measurements of F9600 Technical in CD-1 Mice
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2017)
Date performed	November 2014 – May 2016
Test facility	[redacted]
Report reference	Study no. [redacted]-105120
Guideline(s)	OECD Guideline 451 (2009)
Deviations from the guideline	None
GLP	Yes
Test material	F9600 technical Batch PL14-0049, purity 96 %
Method of analysis	[redacted], *2014;* [redacted]*; Study No.* [redacted]*-105110) and the data are presented in Volume 3, Section CA B.4, Point CA 4.1.2(c).* *The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to the lack of explanation of the use of a non-linear calibration.* However, the method is considered sufficient for regulatory purposes.
Study acceptable	Yes

Material and Methods

In a GLP- and OECD-compliant carcinogenicity study, bixlozone technical was administered *ad libitum* in the diet of Crl:CD1(ICR) mice for 18 months (78 weeks). Administered doses were 250, 1000, and 5000 ppm for both sexes equating to consumed levels of 32, 126, and 647 mg/kg bw/day, respectively, for males and 43, 164, and 834 mg/kg bw/day, respectively, for females. Each group consisted of 50 animals/sex.

Concurrent toxicokinetic groups were treated for 365 days (52 weeks) and each toxicokinetic group consisted of 20 animals/sex. Administered doses were 250, 1000, and 5000 ppm for both sexes equating to consumed levels of 38, 150, 756 mg/kg bw/day, respectively, for males and 50, 202, and 1046 mg/kg bw/day, respectively, for females. Blood samples were collected from 3 animals/sex/toxicokinetic group on Day 1, 14, 28, 90, 180 and 365 days. All surviving toxicokinetic animals were euthanised following the final blood collection (Day 365) and the liver collected to measure liver weight and for possible future histopathological evaluation. In line with the guideline, no clinical-chemistry, haematology and urinalysis investigations were performed.

Results

Survival and clinical signs (Table B 6.5.2.1)

Treatment-related increased incidences of yellow material on the urogenital area and ventral trunk were noted for males in the top-dose group (5000 ppm). These were not considered adverse by HSE. All other observed signs were common for the age and strain of mice, did not show a clear dose-response and occurred in a small number of animals.

The overall survival rates were acceptable in this study (> 50 % after 18 months) across all groups and not affected by treatment. For males the percent survival at the scheduled final necropsy was 74 % (37/50), 82 % (41/50), 86 % (43/50), and 90 % (45/50) in the 0, 250, 1000, and 5000 ppm groups, respectively, and showed a statistically significant increased trend (Kaplan-Meier estimates of survival). For females, the percent survival was similar between groups with 76 % (38/50), 76 % (38/50), 82 % (41/50), and 80 % (40/50) in the 0, 250, 1000, and 5000 ppm groups, respectively.

Mortality was not affected by treatment. The most common causes of death in both sexes across all groups including controls were either undetermined, malignant lymphoma, urinary tract obstruction (males) and chronic

progressive nephropathy (control and 250 ppm female groups only). No single cause of death in the test substance-treated groups exceeded 4 affected mice per group.

Table B 6.5.2.1: Survival in male and female mice administered bixlozone for 18-months

Dose-levels (ppm)	Males				Females			
	0	250	1000	5000	0	250	1000	5000
Initial N, day 0	50	50	50	50	50	50	50	50
Killed for humane reasons	5	5	4	3	4	3	5	3
Found dead	8	4	3	2	8	9	4	7
Total dead	13	9	6	5	12	12	9	9
% mortality	26	18	14	10	24	24	18	20
% survival	**74**	**82**	**86**	**90**	**76**	**76**	**82**	**80**

Palpable masses (Table B 6.5.2.2)

The incidence of palpable masses, number of multiple masses, mean number of masses per animal and mean number of days to the first mass were all unaffected by test substance administration. All values in the test substance-treated groups were generally similar to or lower than those in the control groups.

Table B 6.5.2.2: Summary of palpable mass in the 18-months mice study with bixlozone

	Males				Females			
Dose (ppm)	**0**	**250**	**1000**	**5000**	**0**	**250**	**1000**	**5000**
Dose (mg/kg bw/day)	**0**	**10**	**41**	**217**	**0**	**13**	**53**	**167**
Number of animals in dose group	50	50	50	50	50	50	50	50
Number of animals with masses	6	5	3	1	4	1	2	0
Number of animals with multiple masses	1	1	1	0	0	0	1	0
Mean number of masses per animal	1.2 ± 0.41	1.4 ± 0.89	1.3 ± 0.58	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.00	1.5 ± 0.71	0.0 ± 0.0
Mean number of days to first mass	412 ± 128	462 ± 53	493 ± 43	533 ± 0	427 ± 82	394 ± 0	415 ± 30	0 ± 0

Body weight and body weight gain, food consumption (Table B 6.5.2.3)

There were occasional statistical changes in body weight, body weight gains and / or food consumption observed during the repeated administration of bixlozone; however, the data do not show a clear dose-response relationship. At the top dose of 5000 ppm, the females displayed a fairly consistent (but not statistically significant) decrease in body weight compared to the control group at week 13, 52 and 78 and the percentage of body weight loss increased during the study to reach – 6 % at week 78. A cumulative decrease in body weight changes > 10 % was seen at week 0-13, 0-52 and 0-78, and is considered to be treatment-related and adverse by HSE. At the lower doses of 250 and 1000 ppm the observed differences noted in mean body weight and body weight gain in both males and females could be attributed to biological variability.

Overall treatment-related and adverse body weight changes were observed in females at the top-dose of 5000 ppm (834 mg/kg bw/day), whilst body weights, body weight gains and food consumption were unaffected by bixlozone administration up to the top-dose of 5000 ppm (647 mg/kg bw/day) in males.

Table B 6.5.2.3: Body weight, body weight gain and food consumption for the 18-month oral carcinogenicity study in CD-1 mice

Diet concentration (ppm)	Males				Females			
	0	250	1000	5000	0	250	1000	5000
F9600 intake (mg/kg bw/day	0	32	126	647	0	43	164	834
Mean body weight (g)								
Week 0	30.6 ± 2.23	30.4 ± 2.47	30.2 ± 2.34	30.3 ± 2.40	22.7 ± 1.96	22.9 ± 1.96	23.1 ± 1.74	22.7 ± 1.74
% change from control	-	-0.7	-1.3	-1	-	+0.9	+1.8	0
Week 13	40.3 ± 3.97	40.5 ± 4.35	39.8 ± 3.10	39.5 ± 3.60	30.4 ± 2.61	29.9 ± 3.06	30.4 ± 2.29	29.5 ± 2.42
% change from control	-	+0.5	-1.2	-2	-	-1.6	0	-3.0
Week 52	45.1 ± 5.38	44.8 ± 5.42	44.9 ± 4.65	43.7 ± 4.51	35.0 ± 4.64	34.1 ± 4.20	34.9 ± 3.27	33.2 ± 3.46
% change from control	-	-0.7	-0.4	-3.1	-	-2.6	-0.3	-5.1
Week 78 (18 month)	46.7 ± 5.80	46.0 ± 4.92	46.2 ± 5.09	45.0 ± 6.19	38.5 ± 5.51	37.0 ± 4.93	38.0 ± 4.17	36.2 ± 5.07
% change from control	-	-1.5	-1.1	-3.6	-	-4	-1.3	-6
Mean body weight gain (g)								
Week 0-13	9.6 ± 2.80	10.1 ± 3.08	9.7 ± 2.43	9.2 ± 2.22	7.7 ± 2.34	7.0 ± 1.97	7.3 ± 1.94	6.8 ± 2.02
% change from control	-	+5	+1	-4.2	-	-9	-5.2	-12
Week 0-52	14.5 ± 4.24	14.5 ± 4.38	14.7 ± 4.08	13.4 ± 3.31	12.2 ± 4.51	11.4 ± 3.38	11.8 ± 2.94	10.6 ± 2.84
% change from control	-	0	+1.4	-7.6	-	-6.6	-3.3	-13
Week 0-78	16.1 ± 4.62	15.7 ± 4.31	15.9 ± 4.77	14.9 ± 5.14	15.8 ± 5.65	14.3 ± 3.85	15.1 ± 3.85	13.6 ± 4.41
% change from control	-	-2.5	-1.2	-7	-	-5.3	-4.4	-14
Food consumption (g/animal/week)								
Week 0-1	5.8 ± 0.99	5.8 ± 0.92	5.8 ± 1.03	5.9 ± 1.12	5.6 ± 1.39	6.2 ± 1.50	5.5 ± 1.18	5.4 ± 1.19
% change from control	-	0	0	+1.7	-	+11	-1.8	-3.6
Week 13-14	5.3 ± 1.08	5.4 ± 0.82	5.3 ± 0.69	5.6 ± 1.07	5.3 ± 1.48	5.5 ± 1.81	5.3 ± 1.45	5.4 ± 1.45
% change from control	-	+1.9	0	+5.7	-	+3.8	0	+1.9
Week 51-52	5.4 ± 0.98	5.2 ± 0.71	5.1 ± 0.60	5.1 ± 0.52	5.2 ± 1.14	5.1 ± 1.07	5.0 ± 0.86	4.8 ± 0.93
% change from control	-	-3.7	-5.6	-5.6	-	-1.9	-3.8	-7.7
Week 77-78	4.4 ± 0.87	5.1 ± 5.40	4.4 ± 0.57	3.8 ± 0.84	4.9 ± 0.89	4.5 ± 0.72	4.4 ± 0.66	5.3 ± 6.51
% change from control	-	+16	0	-14	-	-8	-10	+ 8

Clinical pathology – presence of mouse hepatitis virus (MHV) and/or murine rotavirus (epizootic diarrhoea of infant mice; EDIM)

At the request of the applicant, faecal and blood samples were taken from all animals remaining at the time of the terminal necropsy to be analysed using serology and PCR against MHV and ADIM. The tests showed that most samples were positive for MHV (serology and PCR) and EDIM (serology only, negative for PCR). These results indicate that most animals have had both infections at some point during the study.

Since the immune system is not affected by bixlozone, each animal was able to fight off the infection(s). The MHV was likely an enterotropic strain and these strains rarely cause disease – they are not very pathogenic, and they do not spread to other tissues in the body. There were no microscopic changes noted that would be associated with MHV in the liver or gut and none would be expected with EDIM. Also, no bacterial/fungal infections indicative of a compromised immune system were observed. Therefore, the presence of these infections had no impact on the validity of the study.

Toxicokinetics (Table B 6.5.2.4)

There was high variability for bixlozone concentrations found in individual samples, with standard deviations that were near or exceeded the mean for all dose groups on most sampling days. Consistent with the findings from the 90-day repeated-dose toxicity study in mice (**Error! Reference source not found.**) bixlozone mean concentrations were found to be 2- to 13-fold higher in males than in females on most sampling days at all dose levels. Bixlozone mean concentrations tended to be higher on days 14 and/or 28 than on other evaluation days.

Table B 6.5.2.4: Plasma concentration (ng/mL) for bixlozone after dietary administration of bixlozone technical in mice

Males						
Bixlozone (ppm)	**250**		**1000**		**5000**	
mg/kg bw/day	**38**		**150**		**756**	
Study Day	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1	22.5	4.75	17.9	13.6	146	166
14	69.1	99.3	356[a]	561	173	97.2
28	13.7	5.36	63.2	36.7	209	179
90	13.4	8.68	20.1	6.42	78.1	39.4
182	15.2	9.96	33.3	16.1	115	39.8
366	10.7	5.85	42.9	21.6	144	98.6
Females						
Bixlozone (ppm)	**250**		**1000**		**5000**	
mg/kg bw/day	**50**		**202**		**1046**	
Study Day	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1	13.3	11.2	8.2	5.5	128	102
14	10.0	8.7	51.3	52.7	76.4	67.6
28	6.2	2.1	31.5	23.5	193	142
90	BLQ	-	6.5	1.5	34.5	15.5
182	BLQ	-	5.8	1.4	35.3	22.0
366	BLQ	-	8.7	3.8	37.3	29.2

N = 3

BLQ Below the limit of quantitation (5.00 ng/mL). For values assigned as BLQ the most conservative value used was 5.00 ng/mL to estimate the mean plasma concentration unless all samples (n = 3) were assigned as BLQ.

a Mean concentration included high concentration of 1000 ng/mL for 1 male; this concentration was up to 37-fold higher than concentrations in other animals in this group on Day 14. If 1000 ng/mL value is excluded, the mean plasma concentration is 32.3 (no SD with N = 2).

Organ weight changes (Table B 6.5.2.5)

Consistent with repeated-dose toxicity studies in the mouse (**Error! Reference source not found.**), statistically significant changes in liver weights were observed at the top dose for both sexes (5000 ppm). They comprised increased absolute and relative liver weights in females (+21 and +27 % compared to controls, respectively) and males (+19 % and + 22.5 % compared to control males, respectively).

It was pointed out by the study authors that the highest individual animal liver weights seen, regardless of sex or exposure group, were in mice afflicted with primary tumours (hepatocellular adenoma or carcinoma) or systemic tumours (malignant lymphoma or histiocytic sarcoma). For more details please refer to neoplastic findings below. The incidence of these organ weight outliers (since they contain tumours) was noted in the study report to be fairly equally distributed across all exposure groups; therefore, no individual animal organ weights were proposed to be excluded to calculate the mean weights. The approach was considered acceptable by HSE and the results are thus appropriate for comparison purposes.

The ('non-neoplastic') liver weight changes observed in the top dose males were > 15 % compared to controls and were often accompanied with histopathology findings but not in females. Nevertheless, the extent of the liver changes is considered treatment-related and adverse at top-dose for both sexes. The findings are consistent with the parallel plasma kinetics results showing that males are more highly exposed to bixlozone compared to females. No adverse liver effects were seen at the lower dose in males and females (up to 1000 ppm, equivalent to 126/164 mg/kg bw/day in males and females respectively).

Additionally, a dose-related reduction in absolute and relative uterus weights was observed in all treated groups, reaching statistical significance at the top dose. Since organ weights are not normally part of a carcinogenicity study, a historical control database for comparison purposes was not available for this study. Nevertheless, it was reported that the mean uterine weight seen in the control group was considered atypically high owing to highly variable individual data (the standard deviation is higher than the mean value) and a higher incidence in primary uterine tumours and mild to moderate cystic glandular hyperplasia compared to the treated groups (please refer to Tables B.6.5.2.4 and B.6.5.2.5 for corresponding data). Overall, the lower uterine weights seen across all treated groups compared to the control group can be considered incidental and unrelated to test substance exposure. HSE also notes the absence of any uterine tissue-specific histopathology in the treated animals, with findings much more extensive in the controls.

Regarding the thyroid, a dose-related but not statistically significant decrease in absolute weight was observed in males only, reaching – 12 % at 5000 ppm compared to the control group. However, the slight (-9%) relative thyroid weight change in all treated males did not show any dose-related relationship and the opposite effect was seen in females at all doses. Overall the changes seen in the absolute thyroid weight in males are not considered treatment-related or adverse. In addition, no associated histopathology was observed.

There were no other biologically relevant or statistically significant effects on the weights of any other organ.

Overall treatment-related and adverse changes in liver weight were observed in males and females at the top dose of 5000 ppm (647 and 834 mg/kg bw/day in males and females respectively).

Table B 6.5.2.5: Organ weight changes in the 18-month oral carcinogenicity study in CD-1 mice

Diet concentration (ppm)	Males				Females			
	0	250	1000	5000	0	250	1000	5000
F9600 intake (mg/kg bw/day	0	32	126	647	0	43	164	834
Terminal body weight (g)	46.9 ± 5.57	46.2 ± 4.76	46.4 ± 5.03	45.0 ± 6.31	38.1 ± 6.16	36.5 ± 4.91	37.8 ± 4.20	36.5 ± 5.35
Organ weights								
Liver weight (g)	2.25± 0.39	2.12 ± 0.29	2.35± 0.58	**2.69 ± 1.22 ***	1.895 ± 0.81	1.73 ± 0.363	1.79± 0.25	**2.29 ± 0.87 ***
% from control		-6	+4	**+19**		-9	-6	**+21**
Liver/body weight ratio (g/100 g)	4.83 ± 0.796	4.61 ± 0.52	5.08 ± 1.21	**5.92 ± 1.89 ****	4.91± 1.47	4.74 ± 0.75	4.765 ± 0.59	**6.25 ± 1.86 ****
% from control		-5	+5	**+22.5**		-3	-3	**+27**
Uterus (g)					1.43 ± 1.796	0.998 ± 0.85	0.87 ± 0.72	**0.78 ± 0.66***
% from control						-30	-39	**-45**
Uterus/body weight ratio (g/100 g)					3.62 ± 4.17	2.79 ± 2.315	2.34 ± 1.89	2.08 ± 1.66
% from control						-23	-35	-42
Thyroid weight (g)	0.0049± 0.00109	0.0047 ± 0.0024	0.0046± 0.00139	0.0043 ± 0.00121	0.0043 ± 0.00197	0.0044 ± 0.00191	0.0045± 0.00176	0.0041 ± 0.0013
% from control		-4	-6	-12		+2.3	+5	-5
Thyroid/body weight ratio (g/100 g)	0.011 ± 0.0026	0.01 ± 0.005	0.01 ± 0.0031	0.01 ± 0.0031	0.011 ± 0.0051	0.012 ± 0.0058	0.012 ± 0.0058	0.012 ± 0.0037
% from control	-	-9	-9	-9	-	+9	+9	+9

* P<0.05, **P<0.01 determined as statistically significantly different to control using appropriate statistical test

Macroscopic findings (Table B 6.5.2.6)

In females, higher incidences of cervical masses (6/50) and lung nodules (5/50) were seen in the 5000 ppm group compared to the control group (1/50 cervical masses and 0/50 lung nodules).

Because the microscopic examination of the cervix and lung did reveal higher incidences of tumours in the 5000 ppm group, the relevance of these macroscopic incidences (nodules) is discussed in more details below in the Microscopic findings – Neoplastic findings sub-section. Please refer to
Table B 6.5.2.9 for associated data.

Table B 6.5.2.6: Summary of macroscopic findings in the 18-month oral carcinogenicity study in CD-1 mice

	Males				Females			
Dose (ppm)	**0**	**250**	**1000**	**5000**	**0**	**250**	**1000**	**5000**
Dose (mg/kg bw/day)	**0**	**10**	**41**	**217**	**0**	**13**	**53**	**167**
Number of animals in dose group	50	50	50	50	50	50	50	50
Lung mass	3	0	2	2	1	1	1	1
Lung nodule(s)	0	5	2	1	**0**	1	1	**5**
Cervical mass					**1**	0	0	**6**
Cervical nodules					0	0	1	0

Microscopic findings – Non-neoplastic histopathological findings (Table B 6.5.2.7)

The liver microscopic findings from this study correlated well with those of the preceding repeated-dose toxicity studies conducted in the mouse. Minimal to moderate centrilobular or diffuse hepatocellular hypertrophy was noted in all male groups included controls (but not in females), and the incidence increased with the dose from 1000 ppm. The findings also included shape irregularities and cytoplasmic invaginations, variable numbers of mitotic figures, and minimal to mild mixed cell inflammation centering on occasional single cell necrosis. Hypertrophy of hepatocytes was sometimes, but not always, associated with glycogen accumulation (please refer to Table B.6.5.2.5 below).

In males a marginal increased incidence of reduced sperm in the luminal epididymes was seen from the mid dose of 1000 ppm. The incidences (22 % and 24 % at 1000 and 5000 ppm respectively) were still higher that the maximum value (21.7 %) of the laboratory HCD provided, which in addition were not fully compliant with Reg 283/2013 (covering 8 years and with no information on mean and other distribution statistics). Therefore, these findings are considered treatment-related and adverse by HSE.

There was also a marginal increased incidence of pelvis dilation of the kidneys in the top dose males and a marginal increased incidence of minimal chronic glandular inflammation of the stomach from 1000 ppm in males. No such effects were observed in females. Although the incidence of pelvis dilation of the kidney at the top dose (18 %) was within the laboratory HCD provided (21.7 %), these HCD were not fully compliant with Reg 283/2013 (covering 8 years and with no information on mean and other distribution statistics). The HCD provided for the stomach findings showed that the historical control incidence is very low in both sexes, with the incidence in this study from the mid-dose in males being significantly higher that the HCD range. HSE notes that the sex specificity of these findings is consistent with the higher systemic exposure to bixlozone observed in male mice compared to females in this study (see toxicokinetics findings from this study in Table B 6.5.2.4 6.1 and more on toxicokinetics on mice in Section B.6.1.1.3). Overall, HSE concludes that the stomach and kidney findings in males are treatment-related and adverse.

No other treatment-related microscopic findings in any other organ were noted.

Overall a treatment-related and dose-dependent increased incidence in hepatocellular hypertrophy (including glycogen accumulation and single cell necrosis) was noted in males only from 1000 ppm; however, these findings are considered adverse only at top-dose (5000 ppm; 647 mg/kg bw/day) since they correlate with clear and adverse liver weight increases. In addition, slightly higher incidences of reduced epididymal sperm and inflammation of the glandular stomach were seen in males from 1000 ppm, with pelvis dilation of the kidney occurring in males at the top dose of 5000 ppm. Overall, adverse non-neoplastic findings started to occur in males from the mid dose of 1000 ppm (126 mg/kg bw/day).

Table B 6.5.2.7: Non-neoplastic histopathological findings in the 18-month oral carcinogenicity study in CD-1 mice (all types of death combined)

Diet concentration (ppm)	Males (n = 50)				Females (n = 50)			
	0	250	1000	5000	0	250	1000	5000
Bixlozone intake (mg/kg bw/day)	**0**	**32**	**126**	**647**	**0**	**43**	**164**	**834**
non-neoplastic histopathology findings (all animals n = 50)								
Liver								
Hypertrophy, hepatocellular	**4**	**2**	**11**	**18**	**1**	**0**	**0**	**1**
Minimal	3	1	6[a]	7	1	0	0	0
Mild	0	0	1	6	0	0	0	0
Moderate	1[a]	1	4[a]	5[a]	0	0	0	1[b]
% incidence	**8**	**4**	**22**	**36**	**2**	-	-	**2**
Epididymides								
Reduced sperm, luminal	**7**	**7**	**11**	**12**				
Minimal	1	1	none	none				
Mild	2	1	3	3				
Moderate	3	5	6	7				
Severe	1	none	2	2				
% incidence	14	14	**22**	**24**				
HCD (max)[c]	**13 (21.7 %)**							
Kidneys								
Pelvis dilation	4	4	4	**9**	0	0	0	0
Minimal	4	1	4	3	0	0	0	0
Mild	0	3	0	5	0	0	0	0
Moderate	0	0	0	1	0	0	0	0
% incidence	8	8	8	**18**	0	0	0	0
HCD (max)[c]	**13 (21.7 %)**				**-**			
Stomach, glandular								
Chronic inflammation	5	7	**9**	**10**	0	1	2	1
Minimal	5	6	9	10	0	1	2	1
Mild	0	1	0	0	0	0	0	0
% incidence	10	14	**18**	**20**	0	2	4	2
HCD (max)[c]	**2 (3.33 %)**				**1 (2.04 %)**			

a – Associated with increased glycogen deposition
b – Found dead on study day 146
c - HCD (maximum numerical and % incidence) date range: Apr 2009 – May 2017; 8 studies/ 11 control groups; 630 animals

Microscopic findings – Neoplastic findings (Table B 6.5.2.9*)*

There was no increased incidence of benign or malignant liver tumours in both sexes up to the top-dose.

Macroscopically, at the top dose, 6/50 females had cervical masses (compared to 1/50 control) and 5/50 females had lung nodules (compared to 0/50 in controls); please refer to Table B.6.5.2.6 for more details. Microscopically, increased incidences of histiocytic sarcoma, cervical and lung tumours were noted.

Histiocytic sarcoma in females

Increased incidences of histiocytic sarcomas (neoplasia of hematopoietic origin) were seen in top dose females.

The incidence of systemic histiocytic sarcomas in animals, regardless of tissue present within, was 6/50 (12 %) in top dose females vs 2/50 (4 %) in controls, with no clear dose-response since there was no tumours in the mid and low doses. The increased incidence was not statistically significant and was well within the range of the laboratory HCD provided (0.0 – 18.3 %). Although the HCD provided is large (derived from 33 female control groups in 22 studies conducted from August 1999 to May 2017), the data are not compliant with the data requirements laid out in Reg 283/2013 (section 5, point 3) which specify that HCD should "cover a five-year

period, centred as closely as possible on the date of the index study". Nevertheless, these significantly extended HCD can be used in a WoE approach and suggest that the incidence of histiocytic sarcoma in CD-1 female mice is highly variable in controls, with a maximum level of 18.3 %. This data range is also similar to the one reported in Greaves P (2012[5]; 0 - 15 % for CD-1 mice).

According to this review publication, histiocytic sarcoma tumours are uncommon until the age of 12 months, but they increase steeply after 18 months when they are generally more common in females than in males, which is the case in this study. Moreover, the tumour cells are metastatic, and deposits can be found in particular in the lungs and liver. This was also the case in this study, with 3 of the 6 sarcomas being found in the cervix, with metastasis/deposits localised in the lungs and liver.

It is also noted that the biological plausibility of this tumourigenic response is very low as the blood is not a target tissue of toxicity in mice. Moreover, the finding was sex specific, with an increased incidence observed in females only, whilst in males a single incidence was noted at 250 ppm. This sex-specific response is in contrast to the higher systemic exposure to the test substance seen in males compared to females (Table B 6.5.2.4), further reducing the biological plausibility and possible relation to treatment of the finding. Lastly, no similar findings were seen in the combined chronic / carcinogenicity study in rats (Section B.6.5.1) even though these tumours can be relatively common in both species. Finally, it is widely believed that this particular neoplasm lacks relevance in the identification of a human carcinogenic hazard. This neoplasm is very rare in humans and no chemical has been shown to increase the incidence of histiocytic sarcoma in the rat and only rarely in the mouse (Greaves P., 2012).

The PWG panel did not provide a review of these tumour findings.

Overall, considering the sex and species specificity of the response, the low biological plausibility of the finding and the relatively high incidence of this tumour observed in control CD-1 mice in accordance with the laboratory extended HCD supplied and the HCD reported in the available literature, HSE concludes that the incidence in histiocytic sarcomas observed in female mice at the top dose is overall unrelated to treatment with bixlozone.

Cervical tumours in females

Increased incidences of cervical tumours were seen in top dose females.

Two females (4%) of the top dose group showed cervical leiomyosarcoma (malignant tumours) vs none in controls. Significantly extended HCD (1999 – 2017) have been provided by the applicant to help interpret the significance of this response, however these are not compliant with the data requirements laid out for pesticide actives (Reg 283/2013, section 5, point 3) since they do not "cover a five-year period, centred as closely as possible on the date of the index study". These significantly extended HCD (range: 0.0 – 5.8 %) show that the incidence observed in the study is within the incidence range, but significantly higher than the mean incidence value of 0.7 %. The applicant also provided the HCD incidence range of 1.54 - 2.04 % for cervical leiomyosarcoma derived from studies conducted between 2009 and 2017 (i.e performed ± 5 years around the date (2014) of the current study) which confirms that the incidence seen at the top dose in this study is higher than the incidence range. Overall, both sets of HCD show that this tumour type is relatively rare in the mouse and confirm that the observed incidence of 4 % in this study is generally higher than the historical control incidences.

The applicant submitted a review of the incidence of these cervical neoplastic findings by a PWG panel. The panel confirmed the incidences reported in the study report. The panel proposed to combine the incidences of leiomyomas and leiomyosarcomas from the cervix (since the two neoplasms represent a continuum of neoplastic transformation of the smooth muscle cells of the cervix) with those from the uterus because the uterus body adjacent to the cervix can frequently be sampled at necropsy instead of the cervix. This confirmed the increase of cervix/uterus tumours at the top dose compared to controls, but a dose-response was no longer apparent as uterus leiomyomas and leiomyosarcomas were reported also at the low and mid dose. HSE did not find the combination approach acceptable for such a modern study as, microscopically, the pathologist should have been able to distinguish between the uterus and cervix tissues. Therefore, the cervical tumour findings at the top dose reported by the study pathologist and confirmed by the PWG panel remain a concern for HSE.

The concern was forwarded to the applicant and the PWG provided HSE with further clarifications.

The PWG clarified that there are no definitive gross or macroscopic features that clearly demarcate the uterine body (also known as the corpus) from the uterine cervix, and often the uterine corpus and uterine cervix are

[5] Peter Greaves. Histopathology of Preclinical Toxicity Studies. Interpretation and Relevance in Drug Safety Studies. Book • 4th Edition • 2012

collected and identified together. Also, there are no histochemical stains, immunohistochemical stains, or ultrastructural features using transmission electron microscopic examination that can differentiate uterine body smooth muscle cells from uterine cervix smooth muscle cells. Further, the neoplastic lesions arising from the uterine body and/or uterine cervix can frequently obliterate the normal architecture of these tissues and involve both of these regions. As a result, it is not possible to determine if a uterine neoplasm arose in the uterine body or uterine cervix based on gross or microscopic features. On that basis, it is more appropriate to combine the uterine cervical and uterine (horn and body) smooth muscle tumours for analysis.

Overall HSE found that the further clarifications provided on the practical difficulties in isolating the cervix from the uterus in mice and also in differentiating both tissues based on gross, macroscopic and microscopic features especially when neoplasms have developed support the use of the combined incidence of neoplastic lesions from both tissues. More specifically, HSE considered the combined incidence in cervix and uterus leiomyomas separately from the combined cervix and uterus leiomyosarcomas as combination of benign and malignant tumours is not regarded as appropriate by HSE. The combined uterus/cervix tumour incidences presented in the table B.6.5.2.8 below show that a dose-response is no longer apparent for leiomyomas and leiomyosarcomas up to the highest dose tested.

Overall, the cervical/uterine tumours reported in females in this study are not considered to be attributable to exposure to bixlozone.

Table B 6.5.2.8: Comparison and combination of cervix and uterus incidence of leiomyoma and leiomyosarcoma in female mice

Bixlozone diet concentration (ppm)	**0**	**250**	**1000**	**5000**
Bixlozone intake (mg/kg bw/day)	**0**	**43**	**164**	**834**
Cervix (No. Examined)	48	47	48	50
Leiomyoma[a]	1	0	0	1
Leiomyosarcoma[a]	0	0	0	2
Uterus (No. Examined)	50	50	50	50
Leiomyoma	0	1	1	0
Leiomyosarcoma	1	2	0	1
Combined Cervix and Uterus Leiomyomas	1	1	1	1
% incidence	2	2	2	2
Combined Cervix and Uterus Leiomyosarcomas	**1**	**2**	**0**	**3**
% incidence	2	4	0	6

[a] Data for the cervix was updated based on the PWG findings

Lung tumours in both sexes

In the top dose females 5/50 animals had lung nodules compared to 0/50 in controls; the finding appears to correlate well microscopically with a higher incidence in bronchio-alveolar carcinoma seen in top dose females (5/50 – 10 %) compared to the controls (1/50 – 2 %). The increase is not statistically significant and is well within the range of the laboratory HCD provided (0 – 14.3 %). Although these extended HCD (1999 – 2017) are not compliant with the data requirements, HSE notes that the range indicates that the incidence of bronchio-alveolar carcinomas is highly variable in controls, rising up to 14.3 %. It is further noted that there were no increases (rather decreases compared to controls) in bronchio-alveolar adenomas in the top dose females.

In top-dose males there were 4/50 bronchio-alveolar adenomas compared to 1/50 in controls, but only 2/50 bronchio-alveolar carcinomas compared to 3/50 in controls. None of the finding showed a clear dose-relationship or was statistically significant compared to the control group.

Moreover, it is noted that the lung has not been identified as a target organ in the mouse and that there is no clear pattern of pre-neoplastic lesions or progression of benign tumours to malignant tumours at the top dose in both sexes. Thus, the biological plausibility of the finding is considered to be low.

The applicant submitted a review of the incidence of these lung neoplastic findings including the bronchiolo-alveolar hyperplasia findings by a PWG panel. The panel reported significantly different incidences to those included in the study report, with the disappearance of the increase and/or the lack of a dose-response. No clear explanation was provided by the applicant as to why the PWG incidences for these tumours were so different from those of the study pathologist, especially considering that this is a modern study. Therefore, HSE has continued to use the study report incidences to draw conclusions on these lung tumour findings.

Overall, the neoplastic findings observed in the lung in males and females are not considered to be attributable to exposure to bixlozone.

Table B 6.5.2.9: Neoplastic histopathology findings in the 18-month oral carcinogenicity study in CD-1 mice

Diet concentration (ppm)	**Males (n = 50)**				**Females (n = 50)**				**HCD#**
	0	**250**	**1000**	**5000**	**0**	**250**	**1000**	**5000**	
F9600 intake (mg/kg bw/day	**0**	**32**	**126**	**647**	**0**	**43**	**164**	**834**	**Mean % incidence (range)**
Macroscopic findings (all animals n = 50)									
Cervical masses					1	0	0	5	
Lung nodules	0	5	2	0	0	1	1	5	
Incidence of neoplastic findings (all animals n = 50)									
Cervix									
Leiomyoma (benign)					1	0	0	1	**0.43 (0.0 – 2.9)**
% incidence					2	0	0	2	
Leiomyosarcoma (malignant)					**0**	**0**	**0**	**2**	**0.7 (0.0 – 5.8)**
% incidence					**0**	**0**	**0**	**4**	
Liver									
Hepatocellular carcinoma	2	1	3	2	1	0	0	0	
Hepatocellular adenoma	2	1	4	0	0	0	0	0	
Lung #									
Bronchiolo-alveolar hyperplasia	3	0	1	1	2	0	0	1	
Adenoma, bronchiolo-alveolar	**1**	**2**	**1**	**4**	2	1	0	0	**Males 9.85 (0.0 - 17)**
% incidence	**2**	**4**	**2**	**8**	4	2	0	0	
Carcinoma, bronchiolo-alveolar (all)	3	4	3	2	1	1	2	5	
Minimal								2	
Mild				1		1			
Moderate	2	4	3	1			1		
Present					1		1	3	
Unscheduled deaths	2	0	0	1	0	1	1	2	
Scheduled necropsy	1	4	3	1	**1**	**0**	**1**	**3**	**Females 4.3 (0.0 - 14.3)**
% incidence	6	8	6	4	**2**	**2**	**4**	**10**	
1-sided pairwise comparison (p)[a]	***N/D***				**-**	***0.8052***	***0.5207***	***0.0481***	
1-sided trend test (p)[b]					***0.0210***				
Systemic tumours									
Sarcoma, histiocytic (regardless of tissue present within)	**0**	**1**	**0**	**0**	**2**	**0**	**0**	**6**	
Unscheduled deaths		1			1			2	
Scheduled necropsy		0			1			4	
% incidence	**0**	**2**	**0**	**0**	**4**	**0**	**0**	**12**	**Females 6.5 (0.0 - 18.33)**
1-sided pairwise comparison (p)[a]	***N/D***				**-**	***1.000***	***1.000***	***0.0797***	
1-sided trend test (p)[b]					***0.0297***				

Diet concentration (ppm)	Males (n = 50)				Females (n = 50)				HCD#
	0	250	1000	5000	0	250	1000	5000	
F9600 intake (mg/kg bw/day	0	32	126	647	0	43	164	834	Mean % incidence (range)
Uterus									
Leiomyoma (benign)					0	1	1	0	
Polyp, endometrial stromal (benign)					3	0	1	0	
Granular cell tumour (benign)					1	0	0	0	
Leiomyosarcoma (malignant)					1	2	0	1	
Sarcoma, endometrial stromal (malignant)					1	0	0	0	
Granular cell tumour (malignant)					0	1	0	0	
Total uterine neoplastic findings					6	3	2	1	

Laboratory HCD from carcinogenicity studies in CD-1 mice: Dates 1999-2017; for males, number of studies = 23; number of control groups = 34; for females, number of studies = 22; number of control groups = 33)
[a] 1-sided pairwise comparison of 0 ppm with active treatment group
[b] 1-sided trend test including 0 ppm and active treatment groups
Statistical Significance: Rare tumour - $p<0.025$ (trend), $p<0.05$ (pairwise); Common tumour - $p<0.005$ (trend), $p<0.01$ (pairwise)
* Statistically significant at the defined significance level
ND = Not determined
NA = not available

Conclusion

Bixlozone was administered via the diet of male and female Crl:CD1(ICR) mice at 250, 1000 or 5000 ppm for 18 months, in a GLP and guideline compliant study.

Some systemic toxicity was observed in both sexes at 5000 ppm (the highest dose tested); the liver was identified as a target organ in both sexes, with the relative liver weight increased by > 15 % compared to controls; however, a clear association with histopathology was only found in males. Consistent with the findings from previous repeated-dose toxicity studies conducted in the mouse, body weight gains and food consumption were not affected by treatment with bixlozone up to the top dose. Higher incidences of reduced epididymal sperm and inflammation of the glandular stomach were observed in males from 1000 ppm (126 mg/kg bw/day), with pelvis dilation of the kidney occurring in males at the top dose of 5000 ppm.

In conclusion, the LOAEL for systemic toxicity is set at the mid dose of 1000 ppm (126 mg/kg bw/d in males) based on adverse effects on epididymal sperm and stomach (chronic inflammation) in males. The NOAEL for systemic chronic toxicity in the mouse is thus set at 250 ppm (32 mg/kg bw/d in males). The systemic toxicity NOAEL proposed by the applicant is 1000 ppm.

Regarding neoplastic findings, HSE concludes that overall there were no tumours attributable to exposure to bixlozone in this study up to the highest dose tested (5000 ppm).

Overall, the NOAEL for carcinogenicity in the mouse is set at the highest dose tested of 5000 ppm equating to 647 and 834 mg/kg bw/day for males and females respectively, based on absence of carcinogenicity. The NOAEL proposed be the applicant is also 5000 ppm.

[redacted] (2017)

Table B 6.5.2.10: Summary of the 18-month carcinogenicity study in the mouse

Method, Species, test substance Acceptability	Doses	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Main adverse effects
Dietary 18-month **Mouse** (Crl:CD1(ICR mice, males and females) GLP OECD 451 (2009) Deviations: none ([redacted] (2017)) **Acceptable**	F9600 technical Batch PL14-0049 Purity 96 % 0, 250, 1000, 5000 ppm Equivalent to: Males: 0, 32, 126, 647 mg/kg bw/day Females: 0, 43, 164, 834 mg/kg bw/day	*Carcinogenicity* 647 (M) 834 (F) (5000 ppm) *Systemic chronic toxicity* 32 (males) (250 ppm)	*Carcinogenicity* > 647 (M) > 834 (F) (>5000 ppm) *Systemic chronic toxicity* 126 (males) (1000 ppm)	*Non-neoplastic findings* <u>5000 ppm</u> ↓ cumulative body weight gain for F > 10 % *Organ weights* ↑ relative liver weight > 15 %** (both sexes) *Histopathology findings* Hepatocellular hypertrophy: 7/10 at 52 weeks & 79 % incidence at 104 weeks (M) ↑ pelvis dilation of kidney (M) ↑ inflammation of glandular stomach (M) ↑ incidence of reduced sperm in epididymes (M) <u>1000 ppm</u> ↑ inflammation of glandular stomach (M) ↑ incidence of reduced sperm in epididymes (M) *Neoplastic findings* None attributable to exposure to bixlozone up to the highest dose tested (5000 ppm).

B.6.5.3. Summary of carcinogenicity

The carcinogenicity potential of bixlozone administered orally was investigated in the rat and the mouse in two long-term OECD and GLP-compliant toxicity studies: a 2-year combined chronic toxicity/carcinogenicity was conducted in rats and an 18-month carcinogenicity in mice. Additional toxicokinetic measurements were also performed in parallel in both studies.

The main non-neoplastic and neoplastic findings are summarised in

Table B 6.5.3.1 below.

Table B 6.5.3.1: Summary of long-term and carcinogenicity studies with rats and mice

Method, Species, test substance	Doses	NOAEL mg/kg bw/day	Main adverse effects
Dietary 24-month **Rat** (Crl:CD (SD) rats, males and females) GLP OECD 453 (2009) Deviations: none ([redacted] (2017)) ***Acceptable***	F9600 technical Batch PL14-0049 Purity 96 % 0, 250, 1000, 5000/3000 ppm Equivalent to: Males: 0, 10, 41, 217 mg/kg bw/day Females: 0, 13, 53, 167 mg/kg bw/day	*Carcinogenicity* 217 / 167 (5000 / 3000 ppm in M/F) *Systemic chronic toxicity* 41 (M) 53 (F) (1000 ppm)	**Chronic phase – 12 months** 5000/3000 ppm (217 / 167 mg/kg bw/d) ↑ incidence of hair-loss in F ↓ body weight-gain in females (10 %** days 1-344) ↑ liver weight in F (18 %*absolute & 11 %* relative) 1000 ppm & 250 ppm No adverse effects **Carcinogenicity phase – 24 months** *Non-neoplastic findings* 5000/3000 ppm (217 / 167 mg/kg bw/d) Dermal atonia and thin body condition in F ↓ body weight gain for both sexes (9-14%** M and 14-24.5%** F) *Organ weights* ↑ relative liver weight > 15 %** (both sexes) ↓ cholesterol**, albumin*, calcium**, total protein* (F) *Histopathology findings - liver* Hepatocellular hypertrophy: 7/10 at 52 weeks & 79 % incidence at 104 weeks (M) 10/10 at 52 weeks & 74 % incidence at 104 weeks (F) Hepatocellular vacuolation: 7/10 at 52 weeks & 32 % incidence at 104 weeks (M) 10/10 at 52 weeks & 74 % incidence at 104 weeks (F) 1000 & 250 ppm No adverse effects *Neoplastic findings* No treatment-related tumours

Method, Species, test substance	Doses	NOAEL mg/kg bw/day	Main adverse effects
Dietary 18-month **Mouse** (Crl:CD1(ICR mice, males and females) GLP OECD 451 (2009) Deviations: none ([redacted] (2017)) ***Acceptable***	F9600 technical Batch PL14-0049 Purity 96 % 0, 250, 1000, 5000 ppm Equivalent to: Males: 0, 32, 126, 647 mg/kg bw/day Females: 0, 43, 164, 834 mg/kg bw/day	*Carcinogenicity* 647 (M) 834 (F) (5000 ppm) *Systemic chronic toxicity* 32 (M) (250 ppm)	*Non-neoplastic findings* <u>5000 ppm</u> ↑ relative liver weight > 15 % (both sexes) Hepatocellular hypertrophy: 7/10 at 52 weeks & 79 % incidence at 104 weeks (M) ↑ pelvis dilation of kidney (M) ↑ inflammation of glandular stomach (M) ↑ incidence of reduced sperm in epididymes (M) <u>1000 ppm</u> ↓ cumulative body weight gain for F > 10 % *Organ weights* ↑ relative liver weight > 15 %** (both sexes) *Histopathology findings* Hepatocellular hypertrophy: 7/10 at 52 weeks & 79 % incidence at 104 weeks (M) ↑ pelvis dilation of kidney (M) ↑ inflammation of glandular stomach (M) ↑ incidence of reduced sperm in epididymes (M) <u>250 ppm</u> No treatment-related findings. *Neoplastic findings* None attributable to exposure to bixlozone up to the highest dose tested (5000 ppm).

* P<0.05, **P<0.01 determined as statistically-significantly different to control using appropriate statistical test

In the rat, there were no treatment-related tumours identified for both sexes up to the top dose tested in this study. Therefore, the NOAEL for carcinogenicity in the rat is set at the top dose of 5000 / 3000 ppm (167 / 217 mg/kg bw/day in males and females, respectively).

Systemic toxicity was observed at the top-dose in both sexes throughout the study (5000 ppm / 3000 ppm equating 217 / 167 mg/kg bw/day in males / females respectively); body weight and body weight gain were statistically significantly reduced compared to the control groups and, consistent with the findings of the short-term repeated-dose studies, the liver was identified as a target organ. Treatment-related increases in parameters indicative of adverse effects in the liver (serum cholesterol, albumin, calcium, total protein) were observed in the chronic toxicity top-dose females and correlated with the liver weight changes and associated hepatocellular hypertrophy findings observed in these animals. In males, treatment-related effects in the liver were observed from 1000 ppm (and considered adverse at 5000 ppm) and were characterised by liver weight changes at 5000 ppm and hepatocellular hypertrophy findings observed from 1000 ppm.

Overall, the NOAEL for systemic chronic toxicity in the rat is set at 1000 ppm (41 / 53 mg/kg bw/day in males / females respectively) with a LOAEL set at 5000/3000 ppm (217 / 167 mg/kg bw/day in males / females respectively) based on adverse effects observed in the liver (serum chemistry changes, liver weight changes and histopathology findings), effects on body weights in both sexes.

In the mouse, there were no neoplastic findings attributable to exposure to bixlozone up to the highest dose tested (5000 ppm).

Overall, the NOAEL for carcinogenicity in the mouse is set at the highest dose tested of 5000 ppm equating to 647 and 834 mg/kg bw/day for males and females respectively, based on absence of carcinogenicity findings. Some systemic toxicity was observed in both sexes at 5000 ppm (the highest dose tested); the liver was identified as a target organ in both sexes, with the relative liver weight increased by > 15 % compared to controls; however, a clear association with histopathology was only found in males. Consistent with the findings from previous repeated-dose toxicity studies conducted in the mouse, the body weight gains and food consumption were not affected by treatment with bixlozone up to the top dose. Higher incidences of reduced epididymal sperm and inflammation of the glandular stomach were observed in males from 1000 ppm (126 mg/kg bw/day), with pelvis dilation of the kidney occurring in males at the top dose of 5000 ppm.

In conclusion, the LOAEL for systemic toxicity is set at the mid dose of 1000 ppm (126 mg/kg bw/day in males) based on adverse effects on sperm and stomach in males. The NOAEL for systemic chronic toxicity in the mouse is thus set at 250 ppm (32 mg/kg bw/day in males). The systemic toxicity NOAEL proposed by the applicant is 1000 ppm.

The overall/most sensitive **NOAEL for carcinogenicity is set at 217 / 167 mg/kg bw/day (5000 / 3000 ppm in M/F)** with a LOAEL of > **217 / 167 mg/kg bw/day** based on absence of neoplastic findings in the rat 2-year combined chronic toxicity / carcinogenicity study up to the highest dose tested.

The applicant proposed an overall NOEL for carcinogenicity at 5000 ppm for males and females, the highest dose level evaluated in the rat bioassay, corresponding to actual consumed dose levels of 217 and 167 mg/kg bw/day for males and females, respectively.

The overall/most sensitive **NOAEL for chronic systemic toxicity is 32 mg/kg bw/day** (250 ppm) identified for effects on epididymal sperm and stomach inflammation in males in the mouse 18-month carcinogenicity study at the LOAEL of 1000 ppm (126 mg/kg bw/day in males).

The applicant proposed an overall NOEL for chronic systemic toxicity at 53 mg/kg bw/day (1000 ppm) based on reduced body weight gain in females noted at 167 mg/kg bw/day (3000 ppm) in the rat 2-year combined chronic toxicity / carcinogenicity study.

Overall, long term oral administration of bixlozone was not carcinogenic in the rat or mouse. Therefore, classification of bixlozone for carcinogenicity is not required (see aligned MCL report).

The following NOAELs have been identified for the chronic toxicity and carcinogenicity of bixlozone.

Table B.6.6.3.1 Summary of NOAELs from carcinogenicity studies with bixlozone

Study, guideline, reference	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Adverse effects at LOAEL
Dietary 24-month GLP OECD 453 (2009) Deviations: none ([redacted] (2017))	**Rat** (Crl:CD (SD) rats, males and females) 0, 250, 1000, 5000/3000 ppm Equivalent to: Males: 0, 10, 41, 217 mg/kg bw/day Females: 0, 13, 53, 167 mg/kg bw/day	*Carcinogenicity* 217 / 167 (5000 / 3000 ppm in M/F)	*Carcinogenicity* > 217 / 167 (5000 / 3000 ppm in M/F)	*Carcinogenicity* No biologically relevant neoplastic findings
		Systemic chronic toxicity 41 (males) 53 (females) (1000 ppm)	*Systemic chronic toxicity* 217 (males) 167 (females) (5000/3000 ppm)	*Systemic chronic toxicity* Dermal atonia and thin body condition in F ↓ body weight gain for both sexes (9-14%** M and 14-24.5%** F) *Organ weights* ↑ relative liver weight > 15 %** (both sexes) ↓ cholesterol**, albumin*, calcium**, total protein* (F) *Histopathology findings - liver* Hepatocellular hypertrophy: 7/10 at 52 weeks & 79 % incidence at 104 weeks (M)

Study, guideline, reference	Species, doses tested	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Adverse effects at LOAEL
				10/10 at 52 weeks & 74 % incidence at 104 weeks (F) Hepatocellular vacuolation: 7/10 at 52 weeks & 32 % incidence at 104 weeks (M) 10/10 at 52 weeks & 74 % incidence at 104 weeks (F)
Dietary 18-month GLP OECD 451 (2009) Deviations: none ([redacted] (2017))	**Mouse** (Crl:CD1(ICR mice, males and females) 0, 250, 1000, 5000 ppm Equivalent to: Males: 0, 32, 126, 647 mg/kg bw/day Females: 0, 43, 164, 834 mg/kg bw/day	*Carcinogenicity* 647 (M) 834 (F) (5000 ppm)	*Carcinogenicity* > 647 (M) > 834 (F) (> 5000 ppm)	*Carcinogenicity* None attributable to exposure to bixlozone up to the highest dose tested (5000 ppm).
		Systemic chronic toxicity 32 (males) (250 ppm)	*Systemic chronic toxicity* 126 (males) (1000 ppm)	*Systemic chronic toxicity* ↑ incidence of reduced sperm in epididymes (M) ↑ incidence of inflammation of glandular stomach (M)

B.6.6. REPRODUCTIVE TOXICITY

The reproductive toxicity potential of bixlozone has been investigated in a two-generation study in rats and two developmental toxicity studies, one conducted in rats and one in rabbits.

B.6.6.1. Generational studies

An oral (dietary) dose-range finding reproduction study and a modern two-generation study in rats are available.

B.6.6.1.1. *Dose range finding reproduction/developmental study*

A dose-range finding study was conducted to determine the doses to be used for the reproductive toxicity studies in rats.

Study	A Dose Range Finding Oral (Dietary) Reproduction/Developmental Study of F9600 Technical in Sprague-Dawley Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016b)
Date performed	Oct-Dec 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105133
Guideline(s)	None
Deviations from the guideline	N/A
GLP	No
Test material	F9600 technical Batch PL14-0049, purity 96%
Method of analysis	*Validation not required since this is a dose range-finding study.*
Study acceptable	Yes but as supplementary study only since this is a dose-range finding study

Material and Methods

Bixlozone technical (batch PL14-0049, purity 96%) was administered via the diet to male and female Crl:CD(SD) rats (10/sex/group) for 14 days prior to mating (F_0 generation). After the pre-mating period, males continued to receive the test substance throughout mating until sacrifice (28 days total exposure) while females, who were allowed to deliver and rear their pups to weaning on lactation day 21 (or until post-mating day 25 for those that failed to deliver), received the test substance throughout mating, gestation and lactation until sacrifice (57-69 days total exposure).

Doses of 0, 300, 1000 and 3000 ppm in males equated to test substance intakes of 0, 17, 56 and 176 mg/kg bw/day. Initial doses of 0, 300, 1000 and 3000 ppm in females were reduced to 0, 150, 500 and 1500 ppm during lactation to maintain consistent dose levels during anticipated increases in food consumption. The doses administered to F_0 females equated to test substance intakes of 0, 20, 62 and 172 mg/kg bw/day during the pre-mating period, to 0, 22, 74 and 217 mg/kg bw/day during gestation and to 0, 23, 86 and 251 mg/kg bw/day during lactation.

Four pups/sex/litter were randomly selected on the Post-Natal Day 4 (PND 4) as the F_1 generation; these pups were exposed during lactation to milk from dams receiving the reduced test substance intakes (0, 150, 500 and 1500 ppm) and for a further seven days following weaning to the original target concentrations (0, 300, 1000 and 3000 ppm).

Results

Parental toxicity

There were no deaths or treatment-related signs of toxicity. There were no statistically significant, treatment-related changes in the mean body weights or body weight gain of male F_0 animals at any dose tested. In females a statistically significant reduction in the mean body weights of the 3000 ppm dose group (-35 %) at the end of the pre-mating period (days 0-14) was attributed to lower body weight gains at the start of the study (days 0-7); food consumption was also statistically significantly lower than controls during this period. Female body weights tended to be lower than controls during gestation and higher than controls during lactation for the 3000

ppm dose group although changes were not statistically significant. Overall, there were adverse effects on body weights, body weight gain and food consumption in females at the top dose of 3000 ppm.

Liver weights were statistically significantly increased in males at 3000 ppm (+15 % absolute and 19 % relative) and at 1000 ppm (+12 % relative); increases in the liver weights of females at the top-dose were not statistically significant (+14 % absolute and 13% relative). Statistically significant increase in relative kidney weights in males was noted at 3000 ppm (+11 %). The only other statistically significant effect on organ weights was a lower left epididymis weight relative to brain weight at 3000 ppm (-11 %) and lower right epididymis weight relative to brain weight at 3000 ppm (-8 %) and at 1000 ppm (-7.5 %); since there were no statistically significant changes seen for the mean and relative to body weight epididymis weights, HSE does not consider the finding to represent a relevant treatment-related effect. Overall, there were adverse increases in liver and kidney weights in males at the top dose.

Reproductive toxicity

There was no adverse effect on male or female fertility, mating, copulation, and conception indices, the numbers of days between pairing and coitus at any dose tested and compared to the controls; neither was there any effect on mean gestation duration or parturition performance (mean gestation lengths were 21.8, 21.6 and 21.5 days for the 300, 1000 & 3000 ppm groups respectively, compared with 21.8 days in the concurrent control group). The number of implantation sites was similar across the control and all treated groups.

Offspring toxicity

Bixlozone had no effect on the mean number of pups born, litter size, sex ratio or pup survival. Pups (affected litters) were found dead or were euthanized at a rate of 4(3) in the high-dose group compared with 9(4) in the control group and 2(2) in the mid- and low-dose groups. In the control group 6(2) pups (litter) and 2(1) in the low-dose group were missing, presumed cannibalised.

All pups selected for the F_1 generation survived to the scheduled necropsy. The only clinical signs noted were a short stature in two animals (one male at 3000 ppm and one female at 1000 ppm) and a missing tail portion of one female at 3000 ppm; these isolated incidences are unlikely to be treatment-related.

Body weight gains of male and female F_1 pups were statistically significantly lower than controls at 3000 ppm during PD 21-28 to an extent of -5 to -7 % in males and of -6 to -8 % in females. Terminal body weights in the 3000 ppm groups were -10 % lower than controls in females (terminal body weights were also lower in males of this group but without statistical significance); there was no corresponding effect on food consumption. Overall, there were adverse effects on body weight and body weight gain in F1 pups at the top dose.

No treatment-related macroscopic findings were seen in F_1 males and females. Absolute liver weights of the F_1 females were statistically significantly greater than controls at 3000 ppm (+15 %), 1000 ppm (+15 %) and 300 ppm (+10 %) whilst relative liver weights were increased at 3000, 1000 and 300 ppm by 29 %, 15 % and 8.5 % respectively. In males, the relative liver weight increase at 3000 ppm (23 %) was statistically significant. Overall, there were adverse increases in liver weights in offspring from 1000 ppm.

Conclusion

In conclusion, in this reproductive range-finding study in which bixlozone was administered in the diet at 0, 300, 1000 and 3000 ppm, there were no adverse effects on reproductive parameters. Parental treatment-related and adverse effects were observed in the F_0 generation at 3000 ppm comprising lower mean body weights and increased liver and kidney weights. In offspring, adverse increased liver weights were observed in females from 1000 ppm and in males at the top dose. In addition, lower pup body weights were noted at the top-dose of 3000 ppm.

Based on these findings, doses of 0, 150, 750 and 3000 ppm were selected for the succeeding two-generation reproductive toxicity study.

A robust NOAEL could not be set from this dose-range finding study.

[redacted] (2016b)

B.6.6.1.2. *Two-generation reproductive toxicity study*

A modern guideline and GLP compliant two-generation reproductive toxicity study has been conducted in rats.

Study	A Dietary Two-Generation Reproductive Toxicity Study of F9600 Technical in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016c)
Date performed	April 2015 – July 2016
Test facility	[redacted]
Report reference	Study no. [redacted]-105134
Guideline(s)	OECD Guideline 416 (2001) – current guideline
Deviations from the guideline	There were no deviations of significance
GLP	Yes
Test material	F9600 technical Batch PL14-0049; purity 96 %
Method of analysis	*The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to insufficient number of recovery determinations at each fortification level.* However, the method is considered sufficient for regulatory purposes.
Study acceptable	Yes

Material and Methods

Bixlozone technical (batch PL14-0049; purity 96%) was administered to male and female Crl:CD(SD) rats (25/sex/group) at dietary concentrations of 0, 150, 750 and 3000 ppm based on the previous dose-ranging study findings. Treatment started for males and females of the F_0 generation 70 days (minimum) prior to mating. Males continued to receive the test substance throughout mating and females throughout mating, gestation and lactation.

Prior to weaning on PND 21, 25/sex/group offspring were randomly selected as the F_1 generation (each litter had previously been reduced on PND 4 to 4/sex/litter); the males of this generation continued to receive the test substance throughout mating, and the females throughout mating, gestation and lactation until weaning of the F_2 generation. The test substance concentration for the F_0 and F_1 generation females was halved during lactation to account for expected increases in food consumption during this period. Actual mean test substance intakes for males and females during pre-mating, mating, gestation and lactation for the F_0 and F_1 generations are summarised in the table below.

Table B 6.6.1.1:Test substance intakes for the two-generation study

Test substance consumption (mg/kg bw/day)			
Phase of study, generation	**150 ppm**	**750 ppm**	**3000ppm**
Males			
Pre-mating (F_0 males)	10	49	200
Pre-mating (F_1 males)	12	60	238
Post-mating (F_0 males)	7	34	141
Post-mating (F_1 males)	**7**	**34**	**140**
Mean (males)	*9*	*44*	*180*
Females*			
Pre-mating (F_0 females)	11	53	209
Pre-mating (F_1 females)	12	59	241
Gestation (F_0 females)	10	50	203
Gestation (F_1 females)	**10**	**49**	**187**
Lactation (F_0 females)	12	62	261
Lactation (F_1 females)	12	59	255
Mean (females)	*11*	*55*	*226*

Bold values are the most conservative doses to consider when setting the NOAELs and LOAELs

* The test substance concentration for the F_0 and F_1 generation females was halved during lactation to account for expected increases in food consumption during this period

Mating, fertility and conception indices were calculated as follows:

$$\text{Male (Female) Mating Index (\%)} = \frac{\text{No. of males (females) with evidence of mating (or females confirmed pregnant)}}{\text{Total No. of Males (Females) Used for Mating}} \times 100$$

$$\text{Male Fertility Index (\%)} = \frac{\text{No. of Males Siring a Litter}}{\text{Total No. of Males Used for Mating}} \times 100$$

$$\text{Male Copulation Index (\%)} = \frac{\text{No. of Males Siring a Litter}}{\text{No. of males (females) with evidence of mating (or females confirmed pregnant)}} \times 100$$

$$\text{Female Fertility Index (\%)} = \frac{\text{No. of Females with Confirmed Pregnancy}}{\text{Total No. of females used for mating}} \times 100$$

$$\text{Female Conception Index (\%)} = \frac{\text{No. of Females with Confirmed Pregnancy}}{\text{No. of Females with Evidence of Mating (or Females Confirmed Pregnant)}} \times 100$$

Litter parameters were calculated according to the following:

$$\text{Mean Live Litter Size} = \frac{\text{Total Viable Pups on PND 0}}{\text{No. Litters with Viable Pups on PND 0}}$$

$$\text{Postnatal Survival Between Birth and PND 0 or PND 4 (Pre-Selection) (\% Per Litter)} = \frac{\text{Sum of (Viable Pups Per Litter on PND 0 or PND 4 [Pre-Selection]/ (No. of Pups Born Per Litter)*}}{\text{No. of Litters Per Group}} \times 100$$

$$\text{Postnatal Survival for All Other Intervals (\% Per Litter)} = \frac{\text{Sum of (Viable Pups Per Litter at End of Interval N/Viable Pups Per Litter at Start of Interval N)*}}{\text{No. of Litters Per Group}} \times 100$$

Where N = PND 0-1, 1-4 (Pre-Selection), 4 (Post-Selection)-7, 7-14, 14-21, birth -PND 4 (Pre-Selection), or 4 (Post-Selection)-21.

* = Pups that were euthanized due to death of the dam were excluded from pup viability calculations.

Results

Parental toxicity

Clinical observations and survival

There were no treatment-related deaths found for the F_0 generation; one F_0 female was found dead and one was sacrificed during the study; the cause was unknown but as the deaths occurred in the control and low-dose groups only, they were not attributable to treatment with bixlozone.

In the F_1 generation, a female of the mid-dose group was also found dead, but this was attributed to a mechanical head injury.

Observed clinical signs at F_0 and F_1 comprising hair loss on the forelimbs and facial area, decreased defecation and red material around the nose, occurred sporadically in all groups (including the control group) and hence were not considered treatment-related.

Body weight and body weight gains (Table B 6.6.1.2 to Table B 6.6.1.5)

For the females of the F_0 generation, statistically significantly lower mean body weight gains (-15 %) at 3000 ppm compared to controls during pre-mating (study days 0-70) correlated with overall mean body weights for this group that were -5 to -7 % lower than controls at various points throughout this period. Consequently, the mean body weights for this group were -9 % lower than controls at day 0 of gestation and the % difference remained statistically significantly lower than controls until the end of gestation. During lactation the mean

body weights of the top-dose F_0 females recovered owing to body weight gains 94 % greater than controls and reached similar weights to controls (albeit statistically significantly lower) by the end of lactation period. There were no statistically significant effects on the body weight development of F_0 females at or below 750 ppm. In F_0 males, the mean body weight was reduced by 5 % and body weight gain by 6% by day 127 at top-dose and is not considered adverse.

Table B 6.6.1.2:F_0 body weights and body weight gains

Dose Level (ppm)	Males				Females			
	0	150	750	3000	0	150	750	3000
Mean body weight g (% compared to controls)								
Day 0 (% change from control)	194 ± 14.0	194 ± 15.6 (0.0)	193 ± 13.5 (-0.5)	192 ± 14.9 (-1.0)	158 ± 11.4	160 ± 12.1 (+1.3)	159 ± 11.3 (+0.6)	158 ± 12.2 (0.0)
Day 70 (% change from control)	506 ± 47.6	506 ± 56.4 (0.0)	498 ± 45.4 (-1.6)	486 ± 52.9 (-4)	281 ± 20.1	294 ± 24.7 (+5)	278 ± 21.9 (-1.1)	**262 ± 16.0** (-7)**
Day 127 (% change from control)	607 ± 65.0	607 ± 68.0 (0.0)	607 ± 58.4 (0.0)	579 ± 65.2 (-5)	313 ± 21.6	327 ± 23.1 (+4.5)	318 ± 21.8 (+1.6)	**298 ± 18.1* (-5)**
Mean body weight gain g (% compared to controls)								
Days 0-7 (% change from control)	60 ± 6.0	61 ± 7.4 (1.7)	61 ± 6.0 (1.7)	57 ± 6.6 (-5)	27 ± 4.8	31 ± 5.0 (+15)	26 ± 7.7 (-4)	**16 ± 17.1 (-41)****
Days 0-70 Pre-mating period (% change from control)	312 ± 40.7	312 ± 49.1 (0.0)	305 ± 40.5 (-2)	295 ± 47.5 (-5)	122 ± 17.4	134 ± 19.0* (+10)	119 ± 15.9 (-2.5)	**104 ± 12.7** (-15)**
Days 0-127 Entire generation (% change from control)	413 ± 59.6	413 ± 60.3 (0.0)	414 ± 55.5 (0.2)	388 ± 58.6 (-6)	NA	NA	NA	NA

NA Not applicable.
* Statistically significant at 0.05 compared to the control group using Dunnett's test.
** Statistically significant at 0.01 compared to the control group using Dunnett's test.

Table B 6.6.1.3:F_0 female body weights and body weight gains during gestation and lactation

Gestation				
Dosage Level (ppm)	**0**	**150**	**750**	**3000**
Mean body weight g (%compared to controls)				
Gestation Day 0 (% change from control)	287 ± 18.4	296 ± 26.0 (+3)	280 ± 23.4 (-2)	**261 ± 16.7** (-9)**
Gestation Day 20 (% change from control)	424 ± 23.4	**449 ± 34.0 (+6)***	428 ± 38.0 (+0.9)	**387 ± 27.0** (-9)**
Mean body weight gain g (%compared to controls)				
Gestation Days 0-20 (% change from control)	137 ± 17.6	**153 ± 18.4* (+12)**	148 ± 22.8 (+8)	126 ± 20.9 (-8)
Lactation				
Dose Level (ppm)	**0**	**75**	**375**	**1500**
Mean body weight g (%compared to controls)				
Lactation Day 1 (% change from control)	322 ± 22.1	337 ± 27.0 (+5)	321 ± 26.3 (-0.3)	**298 ± 20.3** (-7.5)**
Lactation Day 21 (% change from control)	337 ± 19.3	345 ± 22.7 (+2)	348 ± 23.0 (+3)	331 ± 19.0 (-1.8)
Mean body weight gain g (%compared to controls)				
Lactation Days 1-4 (% change from control)	9 ± 10.8	6 ± 15.1 (-33)	17 ± 7.4 (+88)	16 ± 8.1 (+77)
Lactation Days 1-21 (% change from control)	17 ± 17.6	11 ± 16.3 (-35)	27 ± 15.6 (+59)	**33 ± 14.1** (+94)**

* Statistically significant at 0.05 compared to the control group using Dunnett's test.
** Statistically significant at 0.01 compared to the control group using Dunnett's test.

Mean body weight gain of the high-dose males of the F_1 generation was statistically significantly lower than controls throughout the study (-12 % overall); similarly, the body-weight gain of females of the F_1 generation at this dose was generally lower than controls resulting in lower mean body weights throughout the pre-mating and mating periods. Although body-weight gain recovered during gestation, mean body weights were still consistently lower than controls during this period. Increases in body weight gain during lactation resulted in final mean body weights being similar to controls by the end of lactation. There were no statistically significant effects on the body weight development of F_1 females at or below 750 ppm.

In F_1 males, the mean body weight was reduced by 11 % and body weight gain by 12 % by PND 153 at top-dose of 3000 ppm and is considered adverse at this dose. There were no statistically significant effects on the body weight development of F_1 males at or below 750 ppm.

Table B 6.6.1.4: F_1 body weights and body weight gains

Dose Level (ppm)	Males				Females			
	0	150	750	3000	0	150	750	3000
Mean body weight g (%)								
PND 21 (% change from control)	59 ± 5.1	57 ± 5.1 (-3.4)	58 ± 4.7 (-1.7)	**55± 6.6 (-6.8)***	57 ± 5.3	56 ± 5.5 (-1.8)	56 ± 4.7 (-1.8)	**52 ± 5.6** (-8.8)**
PND 70 (% change from control)	443 ± 31.0	432 ± 27.8 (-2.5)	433 ± 43.3 (-2.3)	**405 ± 39.0** (-8.6)**	246 ± 23.0	247 ± 24.2 (0.4)	252 ± 22.7 (2.4)	232 ± 18.9 (-5.7)
PND 153 (% change from control)	683 ± 61.2	664 ± 53.9 (-2.8)	664 ± 68.3 (-2.8)	**606 ± 66.2 (-11.3)****	318 ± 23.9	319 ± 25.7 (0.3)	321 ± 27.9 (0.9)	**298 ± 22.1* (-6.3)**
Mean body weight gain g (%)								
PND 21-91 Pre-mating period (% change from control)	471 ± 38.1	458 ± 30.7 (-2.8)	459 ± 52.9 (-2.5)	**421 ± 42.9** (-10.6)**	220 ± 20.0	224 ± 26.9 (1.8)	228 ± 28.0 (3.6)	207 ± 20.4 (-5.9)
PND 21-153 Entire generation (% change from control)	623 ± 58.6	606 ± 52.0 (-2.7)	606 ± 66.0 (-2.7)	**551 ± 63.2** (-11.6)**	NA	NA	NA	NA

NA Not applicable.
* Statistically significant at 0.05 compared to the control group using Dunnett's test.
** Statistically significant at 0.01 compared to the control group using Dunnett's test.

Table B 6.6.1.5: F_1 female body weights and body weight gains during gestation and lactation

Gestation				
Dosage Level (ppm)	**0**	**150**	**750**	**3000**
Mean body weight g (%compared to controls)				
Gestation Day 0 (% change from control)	291 ± 21.9	294 ± 27.1 (+1.0)	293 ± 28.6 (+0.7)	**267 ± 22.5** (-8)**
Gestation Day 20 (% change from control)	427 ± 30.2	436 ± 38.5 (+2)	440 ± 36.7 (+3)	**397 ± 32.5* (-7) ±**
Mean body weight gain g (%compared to controls)				
Gestation Days 0-20 (% change from control)	136 ± 14.2	142 ± 22.2 (+4)	147 ± 18.3 (+8)	131 ± 18.7 (-4)
Lactation				
Dose Level (ppm)	**0**	**75**	**375**	**1500**
Mean body weight g (%compared to controls)				
Lactation Day 1 (% change from control)	323 ± 24.5	335 ± 34.3 (+4)	336 ± 39.2 (+4)	302 ± 25.4 (-6)
Lactation Day 21 (% change from control)	349 ± 24.8	351 ± 27.5 (+0.6)	354 ± 27.8 (+1.4)	335 ± 20.5 (-4)
Mean body weight gain g (%compared to controls)				
Lactation Days 1-4 (% change from control)	7± 9.8	3 ± 12.9 (-57)	12 ± 10.7 (+71)	**18 ± 9.8** (+157)**

Lactation Days 1-21 (% change from control)	26 ± 18.6	**16 ± 12.2*** **(-38.5)**	18 ± 15.8 (-31)	32 ± 12.9 (+23)

* Statistically significant at 0.05 compared to the control group using Dunnett's test.
** Statistically significant at 0.01 compared to the control group using Dunnett's test.

Overall a treatment-related and adverse decrease in body weight and body weight gain was observed for females at the top-dose of 3000 ppm in the F_0 generation and in both sexes at the top-dose of 3000 ppm in the F_1 generation.

Food consumption

There were no consistent, statistically significant effects on food consumption in either sex of the F_0 generation at any dose during pre-mating, mating, gestation or lactation. Sporadic increases in food consumption of the F_1 generation during gestation and lactation were not dose-related or statistically significant and therefore were not attributed to treatment with bixlozone.

Macroscopic examination and organ weights findings (Table B 6.6.1.6)

There were no treatment-related macroscopic findings in either the F_0 or the F_1 generations.

Consistent with the 28- and 90-day repeated-dose toxicity studies in rats statistically significant, treatment-related higher liver weights were noted at 3000 ppm in males (+ 13 % absolute and +19 % relative) and in females (+12 % absolute and +18 % relative) of the F_0 generation. In the F_1 generation, increased liver weights were also noted at 3000 ppm in females (+13 % absolute and +21 % relative) and in males (+14 % relative). Associated treatment-related minimal hepatocellular hypertrophy was observed in females of both the F_0 and F_1 generations at top-dose (Table B 6.6.1.7).

Statistically significant increase in relative kidney weights were noted at the top dose in the F_0 generation (+13 % for males and +10 % for females) and in the F_1 generation (+13 % for males and +10 % for females); there were no changes to the absolute kidney weights at any dose tested. Histopathological indications of chronic progressive nephropathy (CPN) were present at 3000 ppm in 11/25 F_0 and 18/25 F_1 males; no increased incidence was observed in females (Table B 6.6.1.7).

A non-statistically significant but dose-dependent increase in the absolute and relative weights of the uterus/cervix/oviducts was also noted in females of the F_0 generation from 750 ppm, reaching + 52 and 58 % for absolute and relative weights at the top-dose of 3000 ppm compared to controls. However, the uterine weight changes seen in the top dose group appeared to be driven by two animals with very low uterus weights, considered to be outliers. If these animals are excluded from the group, the mean uterine weight at the top dose is comparable to controls. Moreover, there were no associated uterine histopathology findings observed in this generation (Table B 6.6.1.7). In the F1 generation, the magnitude of the increase was much less, and no dose-response was evident for the absolute weight. A dose-dependent increase in the incidence of luminal dilation of the uterus was however observed in the F1 generation, reaching statistical significance at the top-dose group. Thus there is no clear correlation between the changes in uterus weight observed in the F_0 generation and the occurrence of uterine luminal dilatation observed in the F1 generation (discussed further under the histopathology heading).

Overall, given the inconsistencies seen between the 2 generations and the high inter-individual variability, HSE consider the uterine weight increases in the F_0 generation at the top dose of 3000 ppm as incidental. The result is consistent with the uterine findings at the top dose of 5000 ppm from the 90-day rat study (Table B 6.3.3.4) which were considered to be not related to treatment and showed high variability between animals.

At the top-dose statistically significant increases in relative brain weights were also noted in males of the F0 generation (+ 5.5 %) and in both sexes of the F1 generation (+ 9.5 and + 6 % in males and females respectively); however, the magnitude of changes is not considered adverse by HSE. Lastly there were sporadic, inconsistent increases (or decreases) in the weight of the spleen seen at the top-dose; HSE does not consider them to be treatment-related effects.

Overall there were adverse liver and kidney weight increases observed for both sexes at the top-dose of 3000 ppm in both generations.

Table B 6.6.1.6: Selected organ weight changes from the two-generation study

Sex	Males								Females							
Generation	F0				F1				F0				F1			
Dosage (ppm)	0	150	750	3000	0	150	750	3000	0	150	750	3000	0	150	750	3000
Organ weights																
Terminal weight (g)	607	608	608	578	701	682	685	**625****	313	326	317	**297***	323	325	329	**302***
% difference from control	-	0.2	0.2	-5	-	-3	-2	**-11**	-	+4	+1.3	**-5**	-	+0.6	+1.9	**-6**
Liver absolute (g)	19.5	19.2	20.3	**22.1****	24.35	22.8	23.6	24.7	11.45	11.	11.92	**12.8****	11.7	11.6	12.4	**13.2***
% difference from control	-	-1.3	+4	**+13**	-	-6	-3	+1.3	-	+1.3	+4	**+12**	-	-1.3	+6	**+13**
Liver relative (g)	3.20	3.15	3.34	**3.82****	3.47	3.33	3.45	**3.95****	3.65	3.57	3.765	**4.32****	3.63	3.565	3.76	**4.38***
% difference from control	-	-1.5	+4	**+19**	-	-4	-0.7	**+14**	-	-2	3	**+18**	-	-1.8	+4	**+21**
Brain absolute (g)	2.20	2.18	2.18	2.15	2.28	2.27	2.27	2.22	1.99	**2.05***	2.04	1.99	2.05	2.08	2.07	2.03
% difference from control	-	-0.9	-0.9	-2.3	-	-0.4	-0.4	-2.6	-	+3	+2.5	0	-	+1.5	+1	-1
Brain relative (g)	0.365	0.36	0.3.63	0.38	0.33	0.33	0.335	**0.36****	0.64	0.63	0.65	**0.67***	0.64	0.64	0.63	**0.67***
% difference from control	-	-0.8	-0.5	+3	-	+2	+2	**+9.5**	-	-0.8	+1.6	**+5.5**	-	+1.1	-0.6	**+6**
Kidneys absolute (g)	4.0	4.0	4.1	4.3	4.6	4.3	4.6	4.6	2.3	2.4	2.45	2.1	2.3	2.35	**2.5***	2.4
% difference from control	-	-0.2	+1.7	+7.5	-	-5	+0.2	+0.2	-	+4	+6	+4	-	+1.3	+7	+3
Kidneys relative (g)	0.66	0.66	0.675	**0.75****	0.655	0.64	0.67	**0.74****	0.74	0.74	0.77	**0.81****	0.72	0.73	**0.76***	**0.73***
% difference from control	-	-0.5	+1.7	**+13**	-	-3	+2	**+13**	-	0	+4.5	**+10**	-	+1	**+6**	**+10**
Spleen absolute (g)	0.87	0.93	0.89	0.92	1.04	1.01	0.95	**0.88****	0.62	0.60	0.60	**0.56***	0.59	0.62	0.61	0.55
% difference from control	-	+7	+2	+6	-	+3	-9	**-15**	-	-3	-3	**-10**	-	+5	+3	-7
Spleen relative (g)	0.14	0.15	0.15	**0.16***	0.15	0.15	0.14	0.14	0.20	0.18	0.19	0.19	0.18	0.19	0.18	0.18
% difference from control	-	+7	+3	**+11**	-	0	-6	-4	-	-7.5	-5	-6	-	+5	+1.1	0
Uterus/cervix/oviducts Absolute (g)									0.73± 0.28	0.71± 0.22	0.87± 0.46	1.11± 1.41	0.70	0.78	0.84	0.78
% difference from control									-	-2.7	+19	+52	-	+11	+20	+11
Uterus relative (g)									0.23	0.22	0.28	0.37	0.22	0.24	0.26	0.26
% difference from control									-	-5	+20	+58	-	+12	+18.5	+20

Relative: to final body weight

* Statistically significant at 0.05 compared to the control group using Dunnett's test.

** Statistically significant at 0.01 compared to the control group using Dunnett's test.

Histopathology findings (Table B 6.6.1.7)

Treatment-related minimal hepatocellular hypertrophy was observed in females of both the F_0 and F_1 generations at the top-dose, where statistically significant, treatment-related higher liver weights were noted at 3000 ppm in males and females of both generations. The finding is consistent with those of the 28- and 90-day repeated-dose toxicity studies conducted in the rat (Section **Error! Reference source not found.**).

Histopathological indications of chronic progressive nephropathy (CPN) were present at 3000 ppm in 11/25 F_0 (vs 6/25 in controls) and 18/25 F_1 males (vs 12/25 in controls); no increased incidence was observed in females. These incidences are well within the range of the laboratory HCD provided and although not fully compliant with the requirements of Reg 283/2013 (they cover 8 years), they are considered acceptable by HSE.

No increased incidence of CPN was observed in the other studies conducted in this strain of rat, such as in the repeated-dose toxicity and carcinogenicity studies (Section **Error! Reference source not found.** & **Error! Reference source not found.**). CPN is known to be a spontaneous renal disease commonly found in the laboratory rat. It is most severe in Sprague Dawley rats, with a distinct male predisposition in respect of onset, incidence, severity and progression. Particularly in males, the disease can progress to end-stage kidney, which is a prelude to death from chronic renal failure (Gray, 1977[6]; Hard and Khan, 2004[7]). CPN can also be found in younger rats (Hard *et al.*, 2013[8]). Hard *et al*., 2009[9] provide a comparison between CPN in rats and nephropathies in humans; this publication concludes that CPN is a rat-specific disease with no counterpart in humans.

Overall, there were no treatment-related or adverse histopathological findings in the male kidney.

In the prostate, mononuclear cell infiltration (chronic inflammation) was evident at 3000 ppm in males of both generations, reaching statistical significance for the F_1 males; the incidence of this finding in the F0 generation is within the range of the laboratory HCD provided, but it is above the range for the F1 generation. In view of this and considering that the HCD are not fully compliant with the requirements of Reg 283/2013 (they cover 8 years), HSE concludes that the prostate inflammation in top dose males is treatment-related and adverse.

In females a statistically significant increase in the incidence of luminal dilation of the uterus was observed in the F1 generation only at the top-dose. Although a dose-response may be apparent, HSE notes that when taking into account the small number of animals subjected to histopathology investigations at the low and mid doses, this cannot be confirmed. The increase at the top dose is well within the laboratory HCD provided and although not fully compliant with the requirements of Reg 283/2013 (they cover 8 years), they are considered acceptable by HSE. Overall, therefore, the finding of luminal dilatation of the uterus at the top dose in the F1 generation is considered unrelated to treatment.

Other sporadic histopathological findings: testicular atrophy (two F_0 750 ppm males who failed to sire a litter) and a high grade oligodendroglioma (one 750 ppm male) were isolated findings that were not dose-related; therefore, HSE considers these to be spurious findings, not related to treatment with bixlozone.

Overall, treatment-related minimal hepatocellular hypertrophy was observed in females of both the F_0 and F_1 generations at the top-dose. In addition, there was prostate inflammation in males of both generations at the top dose.

[6] Gray J. E. (1977). Chronic progressive nephrosis in the albino rat. Crit. Rev. Toxicol. 5, 115–144

[7] Hard G. C., Khan K. N. (2004). A contemporary overview of chronic progressive nephropathy in the laboratory rat, and its significance for human risk assessment. Toxicol. Pathol. 32, 171–180

[8] Hard, Gordon C et al. "Consideration of rat chronic progressive nephropathy in regulatory evaluations for carcinogenicity." Toxicological sciences : an official journal of the Society of Toxicology vol. 132,2 (2013): 268-75.

[9] Hard G. C., Johnson K. J., Cohen S. M. (2009). A comparison of rat chronic progressive nephropathy with human renal disease—implications for human risk assessment. Crit. Rev. Toxicol. 39, 332–346

Table B 6.6.1.7: Selected histopathology findings from the two-generation study

Sex	Males								Females							
Generation	F0				F1				F0				F1			
Dosage (ppm)	0	150	750	3000	0	150	750	3000	0	150	750	3000	0	150	750	3000
Microscopic findings																
Liver (N)	25	0	0	25	25	0	0	25	24	24	25	25	25	25	24	25
Hypertrophy, Hepatocellular	0	0	0	0	0	0	0	0	0	0	0	**18***	0	0	0	**20***
Minimal	-	-	-	-	-	-	-	-	-	-	-	18	-	-	-	20
Mild	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kidneys (N)	25	9	13	25	25	7	6	25	24	1	1	25	25	1	1	25
Chronic progressive nephropathy	6	0	1	**11**	12	2	3	**18**	3	1	0	2	9	0	0	6
Minimal	6	-	1	**10**	10	2	3	**18**	3	0	-	2	8	-	-	6
Mild	-	-	-	1	2	0	0	0	0	1	-	0	1	-	-	-
% incidence	24	0	8	**44**	48	29	50	**72**								
HCD (max)#	**22 (78.6 %)**				**24 (85.7 %)**											
Prostate (N)	25	3	3	25	25	1	0	25								
Mononuclear cell infiltrate	3	1	0	**10 (40 %)**	4	0	0	**12* (48 %)**								
Minimal	2	1	0	7	3	0	0	10								
Mild	1	0	0	3	1	0	0	2								
HCD (max)#	**21 (70.0 %)**				**12 (42.9 %)**											
Uterus (N)									24	5	7	25	25	5	6	25
Dilation, luminal									4	4	4	6	1	3	5	**7* (28 %)**
Minimal									-	1	-	2				
Mild									4	3	2	2	1	3	5	**7***
Moderate									-	-	1	-				
marked									-	-	1	-				
HCD (max)#													**12 (48.0 %)**			

* = Significantly different from the control group at the 0.05 level using 2-tailed fisher's exact test.
# = HCD maximum numerical (incidental) date range: Nov 2011 – Mar 2018; F_0 generation:13 animals/ 13 control groups; 333 animals. F_1 generation: 12 studies/ 12 control groups; 308 animals

Overall parental toxicity comprised of lower mean final body weights and / body weight gain for F_0 females and both F_1 males and females of the 3000 ppm group (top dose) compared to the control group. In addition, higher mean absolute and relative liver weights were noted in both sexes compared to the control groups; since the majority of the relative liver weights exceed +15 % compared to controls, the findings are considered adverse by HSE. The liver weight changes correlated to the observation of minimal hepatocellular centrilobular hypertrophy in the 3000 ppm F_0 and F_1 females. Higher relative kidney weights were noted in males and females at the top dose and were ≥ 10 % compared to the control groups in both generations. Also, prostate inflammation was observed in both generations at the top dose.

In conclusion, the parental general toxicity parameters investigated showed a treatment-related and adverse decrease in mean body weight in both F_0 and F_1 males and females in the 3000 ppm group (top dose) compared to the control group, accompanied by an adverse increase in relative liver and kidney weight, which correlated with histopathology findings for the liver. Also, prostate inflammation was observed at the top-dose.

Reproductive parameters

Reproductive performance (Table B 6.6.1.8 & Table B 6.6.1.9)

There were no treatment-related effects on the number of implantation sites in either generation (differences between treated groups and controls were slight and not statistically significant), neither were there any treatment-related alterations in ovarian primordial follicle counts amongst the ovaries examined.

Male and female mating, fertility, copulation and conception indices were marginally lower at the top dose in the F_0 generation. These differences were not confirmed in the F1 generation and thus are considered unrelated to treatment.

In the control, 150, 750 and 3000 ppm groups of the F_0 generation the number of males that failed to sire a litter were 1, 3, 3 and 4 and the number of females determined to be non-gravid were 1, 3, 3 and 4 respectively. In the same respective groups of the F_1 generation 3, 1, 0 and 1 males failed to sire a litter and 3, 1, 0 and 1 females were determined to be non-gravid. Given the inconsistencies between generations, these changes are not considered treatment-related.

The mean number of days between pairing and coitus and the mean oestrous cycle duration was overall similar across all dose groups including the controls for both F_0 and F_1 generations. Any differences were slight, not statistically significant and not dose-related and therefore they are not considered related to treatment with bixlozone.

Overall there were no test-substance related effects observed on male and female reproductive performance parameters of the F_0 or F_1 generation at any dose.

Table B 6.6.1.8: Male and female reproductive parameters of the F_0 generation

Parameter	Dose Level (ppm)				HC Mean[a] (Range)
	0	150	750	3000	
Male Mating Index (%)	100.0	92.0	96.0	96.0	97.8 (86.7-100.0)
Female Mating Index (%)	100.0	92.0	96.0	96.0	97.8 (86.7-100.0)
Male Fertility Index (%)	96.0	88.0	88.0	84.0	91.3 (70.0-100.0)
Female Fertility Index (%)	96.0	88.0	88.0	84.0	91.0 (70.0-100.0)
Male Copulation Index (%)	96.0	95.7	91.7	87.5	93.4 (70.0-100.0)
Female Conception Index (%)	96.0	95.7	91.7	87.5	93.1 (70.0-100.0)
Oestrous Cycle Length (days)	4.1	4.8	4.2	4.2	4.6 (3.9-7.6)
Pre-Coital Interval (days)	3.0	2.5	3.0	2.7	2.9 (2.2-4.6)

[a] HCD range (Jan 2009 – March 2018); 18 studies/ 34 control groups

Table B 6.6.1.9: Male and female reproductive parameters of the F_1 generation

Parameter	Dosage Level (ppm)				HC Mean[a] (Range)
	0	150	750	3000	
Male Mating Index (%)	96.0	100.0	100.0	100.0	97.8 (86.7-100.0)
Female Mating Index (%)	96.0	100.0	100.0	100.0	97.8 (86.7-100.0)
Male Fertility Index (%)	88.0	96.0	100.0	96.0	91.3 (70.0-100.0)
Female Fertility Index (%)	88.0	96.0	100.0	96.0	91.0 (70.0-100.0)
Male Copulation Index (%)	91.7	96.0	100.0	96.0	93.4 (70.0-100.0)
Female Conception Index (%)	91.7	96.0	100.0	96.0	93.1 (70.0-100.0)
Oestrous Cycle Length (days)	4.2	4.2	4.1	4.1	4.6 (3.9-7.6)
Pre-Coital Interval (days)	3.1	2.8	2.1	3.2	2.9 (2.2-4.6)

[a] HCD range - Jan 2009 – March 2018; 18 studies/ 34 control groups

Gestation length and parturition (Table B 6.6.1.10)

The mean gestation duration of treated groups was similar to that of controls and only slight and non-statistically significant differences were noted, with no evidence of a dose-response. The duration of gestation was 21.7 days in all treated groups of the F_0 generation compared with 21.9 days in the concurrent control group. In the F_1 generation the duration of gestation was 21.8, 21.6 and 21.7 days in the 150, 750 and 3000ppm groups respectively compared with 21.8 days in the concurrent control group. No signs of dystocia were noted in either generation.

Table B 6.6.1.10: Gestation length in F0 and F1 generations

Generation	F0				F1			
Dose (ppm)	0	150	750	3000	0	150	750	3000
Gestation length (days)	21.9	21.7	21.7	21.7	21.8	21.8	21.6	21.7

Overall there were no treatment-related effects on the mean duration of gestation at any dose level in either the F_0 or the F_1 generation.

Spermatogenic endpoints

There were no notable or statistically significant effects on mean testicular and epididymal sperm numbers, rates of sperm production, motility or sperm morphology for either the F_0 or the F_1 generation.

Offspring toxicity

Litter data

Parental exposure to bixlozone had no effect on the mean number of pups born, live litter size or sex ratio; any differences from the control group were slight and were neither dose-related nor statistically significant.

Pup survival was unaffected by treatment with bixlozone for both F_1 and F_2 generation pups at all dose-levels and there were no treatment-related clinical signs of toxicity (Table B 6.6.1.11). In the F_1 pups, the number of foetuses (litter) that were found dead was 13(9), 11(5), 4(4) and 1(1) and those presumed cannibalised were 7(7), 3(3), 0(0) and 2(2) in the 0, 150, 750 and 3000 ppm dose-groups respectively. Pups of the F_2 generation found dead amounted to 8(6), 9(7), 23(9) and 7(5) and 14, 5, 4 and 7 pups from the same respective groups were missing and presumed cannibalised. No internal findings to attribute these deaths to parental exposure of bixlozone were noted at necropsy. In addition, it is noted that deaths (including cannibalised pups) were higher in controls.

Table B 6.6.1.11: Pup survival data from F_1 and F_2 litters (% per litter)

Litter	F1				F2			
Dose (ppm)	**0**	**150**	**750**	**3000**	**0**	**150**	**750**	**3000**
Birth to PND 4 (pre-selection)	95.7 ± 6.2	95.1 ± 12.5	98.8 ± 2.8	98.7 ± 3.1	93.5 ± 8.1	96.5 ± 4.5	93.4 ± 16.7	96.8 ± 5.9
PND 4 (post-selection) to PND 21	97.8 ± 8.1	98.8 ± 5.5	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	99.0 ± 3.5	99.0 ± 3.5	98.4 ± 4.22

Body weight development

The body weight development of pups of the F_1 generation was unaffected by treatment; however lower (compared with controls) body weight gain was noted in both sexes of the 3000 ppm group of the F_2 generation during PND 4-7 (-10 %) and PND 7-14 (-6 %) resulting in lower overall mean body weights for this dose-group on PND 14 (-8 %). There were no statistically significant effects on the body-weight development of F_2 pups at the low- or mid-doses.

Overall neonatal toxicity was observed at the top dose of 3000 ppm. Lower mean F_2 pup body weight gains were noted in the 3000 ppm group during PND 4-14 resulting in mean pup weights that were up to 8 % lower than the control group on PND 14.

Necropsies

Pups selected for organ weight investigation

Pups of the F_1 and F_2 generations that had been selected for organ weight investigations were necropsied after weaning. Consistent with the parental toxicity findings (and previous repeated-dose toxicity studies), higher relative liver weights (and generally statistically significant) were noted in the F_1 generation male and female weanlings of the 3000 ppm dose group (+18 % in males +9 % in females) and in F_2 females of the same dose group (+10 %). No other organs were affected including kidneys and the uterus. Overall, adverse increases in liver weights were seen in F1 male pups.

In the F_1 generation, a misshapen liver was observed in one 3000 ppm pup and a dilated renal pelvis was noted in one 750 ppm pup; there were no other internal findings in any pups of this generation. A dilated renal pelvis was also noted in one pup of each of the 150, 750 and 3000 ppm dose groups of the F_2 generation pups. Further internal findings in the F_2 generation (at 3000 ppm) comprised a small thymus in one pup and a dark red area on the thymus of another. Other findings were limited to the control group only. These isolated findings were not considered treatment-related.

Pups not selected for organ weight investigation

In addition to the pups selected for organ weight analysis, necropsies were also carried out on non-selected pups that were found dead or were sacrificed on PND21 (after the death of the dam). In the F_1 generation findings were generally confined to the control group, excepting a yellow coloured firm liver in one pup at 150 ppm and a red discoloration of the lungs in another of the same group. Of those F_1 pups that were sacrificed following the death of a dam, the only internal findings were dilated renal pelvis in one pup each of the control and 750 ppm groups.

Of the F_2 generation pups found dead, one pup of the 150 ppm dose group displayed renal papillae (not fully developed) and a pup at 750 ppm presented with dark red contents of the abdominal cavity. One pup of the 150 ppm dose group was found to have various skeletal and visceral malformations and developmental variations which were isolated to this one animal, with the exception of a blood vessel variation (no brachiocephalic trunk) which was also noted in one pup of the 3000 ppm group. The only other developmental variations were a distended trunk of two pups of the 150 ppm group. Of the F_2 pups that were sacrificed on PND 21, the only internal findings were yellow areas of the liver (at 750 ppm), a dilated renal pelvis (at 750 ppm) and dilated lateral ventricles in the brain (3000 ppm). No other internal findings were noted.

Overall, the necropsies conducted in pups of the F_1 and F_2 generation did not show any internal findings that could be associated with exposure to bixlozone due to their isolated nature and lack of dose-response.

Developmental landmarks (Table B 6.6.1.12)

The age of attainment of balano-preputial separation, and the mean body weights of F1 male pups at the age of attainment were unaffected by treatment with bixlozone. However, the age of attainment of vaginal opening of F1 pups was statistically significantly greater at 3000 ppm compared to the corresponding control (33.6 days compared with 31.7 days). The mean body weights of the female pups at the age of attainment were unaffected by treatment with bixlozone, which indicates that the delay in vaginal patency was the consequence of reduced pup body weight development, because, once the pup body weight was similar to that of the controls, vaginal opening was attained. Moreover the values seen at the top-dose were well within the laboratory HCD provided, although these cover a period of 10 years. In addition, there were no notable effects on other developmental landmarks and these females went on to mate successfully and produce the F2 generation. Overall, HSE considers this finding the secondary consequence of reduced post-weaning female pup body weight development and not a specific reproductive effect of bixlozone.

Overall, the developmental landmarks investigated in the F1 generation were unaffected by exposure to bixlozone in all test substance-exposed groups.

Table B 6.6.1.12: Developmental landmarks of F_1 pups

Dose level (ppm)	**0**	**150**	**750**	**3000**	**0**	**150**	**750**	**3000**	**HCD Mean (range)**
Developmental landmark	**Males (balano-preputial separation)**				**Females (vaginal patency)**				
Mean age at attainment (days)	44.1	44.5	44	45.6	31.7	32.4	32	33.6**	33.1 ± 0.92 (31.2 - 34.4) N = 18
Mean body weight at age of attainment (g)	252.1	246.6	246.7	240.5	115.6	120.1	118.1	117.3	115.0 ± 6.42 (100.5 / 126.8 N = 18

HCD range - Jan 2009 – Mar 2018; Number of studies: 18 studies/ Number of control groups: 34 control groups

Conclusion

The reproductive toxicity potential of bixlozone was investigated in a modern GLP and guideline-compliant two-generation study in the rat in which the substance was administered in the diet at 0, 150, 750 and 3000 ppm.

Parental toxicity was noted in both sexes and both generations at the top-dose tested of 3000 ppm and was characterised by a decrease in body weight and body-weight gain and an increase in liver and kidney weights which correlated with histopathology findings for the liver. Similar effects have been observed in rats in previous repeated-dose toxicity studies. In addition, mononuclear cell infiltration (chronic inflammation) in the prostate was observed in both generations.

Bixlozone had no effect on male or female fertility or reproductive performance; gestation duration and spermatogenic endpoints were also unaffected by treatment. There was also no effect on litter size, sex ratio, pup survival and developmental landmarks. A delay in vaginal opening was seen in F1 pups at 3000 ppm (33.6 days compared with 31.7 days) whilst mean body weights of these female pups at the age of attainment were unaffected by treatment with bixlozone. Therefore, it can be concluded that the delay in vaginal opening was the secondary consequence of reduced post-weaning female pup body weight development and not a specific reproductive effect of bixlozone.

Consistent with the parental toxicity, body weights and body weight gain of pups in the F_2 generation were affected by treatment with bixlozone at the top-dose, whilst relative liver weights were adversely increased in male pups of the F_1 generation.

Overall, HSE has concluded that the administration of bixlozone to rats in this two-generation study did not have a specific effect on fertility, reproduction, pregnancy outcome or pup survival up to the top-dose tested of 3000 ppm. Therefore, a NOAEL of 3000 pm (the highest dose tested; 140/187 mg/kg bw/day in M/F) is proposed for effects on reproduction. The findings of prostate inflammation were insufficient in nature and severity to cause a functional effect on fertility and reproductive performance but are accounted for by the parental NOAEL.

A NOAEL of 750 ppm (34 / 49 mg/kg bw/day in M/F) for general, parental toxicity is proposed by HSE based on decreased body weights and body-weight gain, increased liver and kidney weights with associated histopathological findings for the liver and mononuclear cell infiltration (chronic inflammation) in the prostate in both generations at the LOAEL of 3000 ppm.

For offspring toxicity, a NOAEL of 750 ppm (34 / 49 mg/kg bw/day in M/F) is proposed by HSE, based on decreased body weights and body weight gain of the F_2 pups and increased liver weights in F1 male pups at the LOAEL of 3000 ppm during the pre-weaning period.

These values are consistent with those proposed by the applicant.

[redacted] (2016c)

Table B.6.6.1.1 Summary of generational studies in rats

Method, species, test substance	Doses	NOAEL/LOAEL	Main effects
Dose range finding study, dietary **Rats**, Crl:CD(SD), males & females, 10/sex/group F9600 technical, batch PL14-0049 Purity: 96 % Vehicle: acetone Not to guideline GLP: No [redacted] (2016b) **Supplementary**	0, 300, 1000, 3000 ppm (0, 150, 500, 1500 ppm during lactation) Equivalent to: Males: 0, 17, 56 and 176 mg/kg bw/day Females: 0, 20, 62 and 172 mg/kg bw/day (pre-mating), 0, 22, 74 and 217 mg/kg bw/day (gestation) & 0,	A NOAEL was not set from this dose-range finding study	***Parental (systemic) toxicity*** ***F_0 generation*** **3000 ppm** ↓ body weight (-35 %, pre-mating) body weight gain & food consumption in F *Organ weights* ↑ liver weight in M (+ 15 % absolute & +19 % relative) ↑ relative kidney weight in M (+11 %) **1000 ppm & 300 ppm** No adverse effects observed.

Method, species, test substance	Doses	NOAEL/LOAEL	Main effects
	23, 86 and 251 mg/kg bw/day (lactation)		***F1 generation*** **3000 ppm** ↓ body weights in F (-10%) ↑ relative liver weights (+29 % F & +23 % M) ↑ absolute liver weights in F (+15 %) **1000 ppm** ↑ relative liver weights in F (+15 %) ↑ absolute liver weights in F (+15%) **300 ppm** No adverse effects observed.
Two-generation reproductive toxicity study, dietary **Rats**, Crl:CD(SD), males & females, 25/sex/group F9600 technical, batch PL14-0049 Purity: 96 % Vehicle: acetone GLP Guideline: OECD 416 (2001) Deviations: none [redacted] (2016c) **Acceptable**	0, 150, 750 & 3000 ppm (reduced to 0, 75, 375 & 1500 ppm during lactation) Equivalent to (mean): Males: 0, 9, 44 & 180 mg/kg bw/day Females: 0, 11, 55 & 226 mg/kg bw/day	**Parental toxicity** **NOAEL: 750 ppm (34/49 mg/kg bw/d in M/F)** **LOAEL: 3000 ppm (140/187 mg/kg bw/d in M/F),** based on decreased body weights and body weight gain, increased liver and kidney weights with associated histopathological findings, prostate inflammation and uterine luminal dilation. **Reproductive toxicity** **NOAEL: 3000 ppm (140/187 mg/kg bw/d in M/F)** No specific adverse effects observed up to the top-dose **Offspring toxicity** **NOAEL: 750 ppm(34/49 mg/kg bw/d in M/F)** **LOAEL: 3000 ppm (140/187 mg/kg bw/d in M/F),** based on decreased body weights and body weight gain of the F2 pups and increased liver weights in F1 male	**Parental (systemic) toxicity** ***F0 generation*** There were no treatment related deaths or clinical signs of toxicity **3000 ppm** ↓ body-weight gains in F (-14 %** days 0-70) ↓ mean body weights in F (-9 %** at gestation) *Organ weights* ↑ absolute liver weights: +13 %** (M) & +12%** (F) ↑ relative liver weights: +19 %** (M) & +18 %** (F) ↑ relative kidney weights: +13 %** (M) & +10 %** (F) *Histopathology* Hepatocellular hypertrophy in F (18/25; minimal) ↑ mononuclear cell infiltration (chronic inflammation) in the prostate **750 & 150 ppm** No adverse effects ***F1 generation*** **3000 ppm** ↓ body-weight gains in M** & F ↓ body weights in M** & F* *Organ weights* ↑ absolute liver weights in F (+13 %**) ↑ relative liver weights: +21 %** (F) ↑ relative kidney weights: +13 %** (M) & +10 %** (F) *Histopathology* Hepatocellular hypertrophy in F (20/25; minimal)

Method, species, test substance	Doses	NOAEL/LOAEL	Main effects
		pups at during the pre-weaning period	↑ mononuclear cell infiltration (chronic inflammation) in the prostate* **750 & 150 ppm** No adverse effects **<u>Reproductive toxicity</u>** No specific treatment-related adverse effects **<u>Offspring toxicity</u>** **3000 ppm** *F1 pups* ↑ relative liver weights: +18 % (M)* *F2 pups* ↓ pup body weight-gain (PND 4-7 & 7-14) ↓ pup body weights (PND 14) **750 & 150 ppm** No adverse effects

B.6.6.2. Developmental toxicity studies

The developmental toxicity of bixlozone has been investigated in rats and rabbits. An oral range-finding study for each species is also available.

B.6.6.2.1. *Range-finding developmental toxicity study in rats*

An oral (gavage) dose-range finding pre-natal developmental toxicity study is available in rats.

Study	An Oral (Gavage) Dose Range-Finding Prenatal Developmental Toxicity Study of F9600 in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016d)
Date performed	April 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105129
Guideline(s)	Non-guideline
Deviations from the guideline	N/A
GLP	No
Test material	F9600 Technical Batch PL14-0049 Purity 96.0 %
Method of analysis	*Validation not required since this is a dose range-finding study.*
Study acceptable	Yes, but as supplementary information only since this is a dose-ranging finding study.

Material and Methods

In a pre-natal developmental toxicity range-finding study, female Crl:CD(SD) rats (8/group) received10 mL/kg of bixlozone via gavage once daily during gestation days (GD) 6-19 at concentrations of 0, 25, 75, 225 and 675 mg/kg bw/day; the doses were selected based on the results of the previous range-finding study in rats ([redacted] 2016b). The vehicle used was 0.5 % [w/v] carboxymethylcellulose (CMC) in 5 % Tween® 80.

Results

Parental toxicity

Clinical observations and survival

There was one death; a female at 675 mg/kg bw/day was sacrificed on GD 11 following marked body-weight loss (-15 %) from GD 6 and reduced food consumption from GD 8. Clinical signs comprised a red/yellow material on the body. Relation of this death to treatment cannot be excluded. Of the surviving animals, the same red/yellow material was observed from GD 8 on the body surfaces of the treated groups and continued to be observed throughout the dosing period. Other clinical signs such as body-hair loss and clear material around the mouth were not persistent and /or occurred at similar frequencies in controls.

Body weight, body weight gains and food consumption

The mean maternal body weight development was affected by treatment with bixlozone in the top-dose group only (675 mg/kg bw/day). Statistically significantly lower mean body weight on GD 6-9 and lower mean body weight gain on GD 9-12 were observed for this dose-group compared to control; despite an increase in body weight gain of 40 % during GD 12-15 the overall maternal body weight gain was decreased by 36 % when compared with the control group; the difference was statistically significant. With regard to net body weights (body weight on day 20 exclusive of the uterus and its contents), mean body weight was -7.5 % lower and body-weight gain was -45 % lower than controls (statistically significant) in this dose-group, whilst the gravid uterine weight was -9 % lower (although not statistically significant). A corresponding lower mean foetal body weight was noted for this group (please refer to foetal data for details).

Mean food consumption in the top-dose group was also statistically significantly increased or decreased at various time points throughout gestation, in a manner corresponding to the alterations observed in body weight and body weight gain; this culminated in a mean food intake that was 18 % lower than the control group when the entire gestation period was evaluated (GD 6-20).

At 225 mg/kg bw/day an initial statistically significant 3 % body weight loss was observed (GD 6-7) compared to the control group. Mean body weight, body weight gain and gravid uterine weight for this dose group were similar to controls with only slight, non-statistically significant differences. Similarly, mean body weights, body-weight gains, net body weight, net body-weight gains and gravid uterine weights of the lower dose groups were not affected by treatment.

On GD 6-7 food consumption was statistically significantly lower than controls in this group and corresponds well to the initial mean body weight loss seen during this time period.

There was no effect on food consumption in the lower dose groups.

Overall significant body weight changes and corresponding reduced food consumption were noted at 675 mg/kg bw/day (top-dose), with an initial weight loss (and reduced food consumption) observed also at 225 mg/kg bw/day.

Macroscopic examinations and organ weight findings

The only macroscopic findings were found in the female killed *in extremis* on GD 11. These comprised dark red areas on the lungs, a white area on the kidneys and red and yellow matting on various body surfaces. The female had 16 normally developing implantations *in utero*. No macroscopic findings were noted in any animal at the scheduled necropsy (day 20).

Mean absolute liver weights were statistically significantly higher than controls at 225 (+17 %) and at 675 mg/kg bw/day (+52 %) whilst relative liver weights (to net body weight) were 17 % higher at 225 mg/kg bw/day and 64 % higher at 675 mg/kg bw/day. Liver weights at 25 and 75 mg/kg bw/day were similar to controls. Overall, adverse increases in liver weight were observed from 225 mg/kg bw/day.

GD 20 laparohysterectoctomy data

Laparohysterectoctomy data on GD20 revealed that there was no effect on intrauterine survival at any dose. Bixlozone did not have an effect on post-implantation loss, live litter size or sex-ratio; furthermore, the mean

number of corpora lutea and implantation sites and the mean litter proportions of pre-implantation loss were similar to controls across all treated groups.

Foetal data

Foetal body weights

Mean foetal body weights in the 675 mg/kg bw/day group were lower than controls to an extent of 3.5 g (-8 %) for males, 3.3 g (-8 %) for females and 3.4 g (-8 %) for combined sexes. Mean foetal body weights were similar to controls at the lower doses.

Morphological data

The numbers of foetuses (litters) available for morphological evaluation were 117(8), 118(8), 120(8), 118(8), and 100(7) in the control, 25, 75, 225, and 675 mg/kg bw/day groups, respectively.

Malformations were observed in 0(0), 0(0), 1(1), 0(0), and 1(1) foetuses (litters) of these same respective dose groups and were considered to be spontaneous in origin. An external malformation was noted in a single foetus of the 75 mg/kg bw/d group, specifically a gastroschisis (a portion of the liver and several loops of intestine protruded through an opening in the ventral midline). In the absence of a dose-response, this finding was considered unrelated to treatment. In the top-dose-group, a soft tissue development malformation was noted in a foetus which was reported to be a retroesophageal arch. This finding was not reproduced in the main study and thus was considered a chance finding unrelated to treatment.

Other soft tissue development variations (pale spleen or distended ureter) were found in control foetuses only; one renal papilla, not fully developed, was also found in one foetus of the control group.

Overall there were no test-substance related morphological changes observed in any of the treated groups compared to the controls.

Conclusion

In a developmental range-finding study, administration of bixlozone to rats throughout gestation resulted in maternal toxicity at the highest dose tested (675 mg/kg bw/day), characterised by 1 death, body weight loss, reduced body weight gain and increases in absolute and relative liver weights. Initial body weight loss and increased liver weight were also observed at 225 mg/kg bw/day.

No external or visceral malformations or variations that were attributable to treatment with bixlozone were apparent but foetal weight was reduced at the top dose.

Based on the findings of this study, doses of 75, 225 and 550 mg/kg bw/day were selected for a definitive pre-natal developmental toxicity study.

[redacted] (2016d)

B.6.6.2.2. *Pre-natal developmental toxicity study in rats*

An oral (gavage) developmental toxicity study is available in rats.

Study	An Oral (Gavage) Prenatal Developmental Toxicity Study of F9600 in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016e)
Date performed	July 2014 – January 2015
Test facility	[redacted]
Report reference	Study no. [redacted]-105130
Guideline(s)	OECD Guideline 414 (2001)
Deviations from the guideline	The protocol states that laparohysterectomies and macroscopic examinations will be performed blind to treatment group. From 18th to 22nd August 2014, the gestation day 20 females send to necropsy for laparohysterectomies were not blinded to treatment group. This deviation is minor and does not compromise the validity of the study. Otherwise there were no deviations of significance.
GLP	Yes
Test material	F9600 Technical Batch PL14-0049 Purity 96.0 %
Method of analysis	*Validation not required since the oral method of administration used was gavage.*
Study acceptable	Yes

Material and Methods

In a pre-natal developmental toxicity study, female Crl:CD(SD) rats (25/group) were administered bixlozone technical (batch PL14-0049, purity: 96%) at doses of 0, 75, 225 and 550 mg/kg bw/day. The test substance was administered as a 10 mL/kg gavage dose, once daily throughout gestation days (GD) 6-19; the concurrent control group received the same volume of vehicle (0.5 % [w/v] CMC in 5 % Tween® 80) under identical experimental conditions.

Results

Maternal toxicity

Clinical observations and survival

There were no deaths, but clinical signs of toxicity similar to those observed in the dose-range finding study were evident, comprising red, yellow and/or clear material on various body surfaces up to one hour after test substance administration (in the mid- and high-dose groups). The effect showed a clear dose-response and occurred from GD 7 throughout the entire dosing period. One female of the 550 mg/kg bw/day dose group presented with hair loss on the ventral trunk and urogenital area from GD 11.

Other clinical signs of toxicity (scabbing) were not dose-related and were also noted in the control group at similar frequencies.

Overall, clinical signs of toxicity were noted from the mid dose (225 mg/kg bw/day).

Body weight, body weight gains and food consumption (Table B 6.6.2.1)

Bixlozone had an effect on maternal body weight development at 550 and 225 mg/kg bw/day. In the high-dose group, body weight gains at the start of dosing (GD 6-9) were 90 % lower than controls and animals lost weight on GD 7 and 8; although body weight gain recovered to be similar to controls on GD 9-12 and 12-5, a further decline in gain of 11 % near the end of the gestation period (GD 15-20) resulted in an overall body weight gain for the entire gestation period (GD 6-20) being 16 % lower than controls (differences are statistically significant). By the end of gestation mean body weights were 6 % lower than controls for this dose group. Net body weight and net body weight gain (excluding the uterus and contents) was -7 and -29.5 % lower than controls for this dose group; mean gravid uterus weights, however, were unaffected by treatment with bixlozone.

In the mid-dose group (225 mg/kg bw/day), an initial reduction in body weight gain at the start of dosing (-40 % for GD 6-9, statistically significant compared to control group) recovered thereafter so that the mean body weight and mean body weight gain were comparable with controls for the remainder of gestation. There were

no effects on either the net body weight or net body-weight gain, or the gravid uterus weight at this dose. Maternal body weight development was not affected by bixlozone administration at the low-dose.

Food consumption was statistically significantly lower than controls in the 550 and 225 mg/kg bw/day dose groups at various time points throughout the dosing period and resulted in an overall food consumption (for GD 6-20) that was 8 % lower than controls in the 225 mg/kg bw/d dose group and 11.5 % lower in the 550 mg/kg bw/d dose group. The lower food consumption noted for the mid- and high-dose groups generally corresponded to the lower body weights at these doses and is considered by HSE to be treatment-related and adverse.

Overall a treatment-related effect on maternal body weight and body weight gain (particularly severe during the first 3 days of dosing) and food consumption was observed at 550 and 225 mg/kg bw/d in this study.

Table B 6.6.2.1: Body weight changes and food consumption in the rat developmental toxicity study

Dose (mg/kg bw/d)	0	75	225	550
Maternal bodyweight (g) day 0	256 ± 13.9	257 ± 14.9	258 ± 15.5	255 ± 12.5
Maternal bodyweight (g) day 6	294 ± 16.2	289 ± 19.5	294 ± 18.8	290 ± 17.
Maternal bodyweight (g) day 7 (% from control)	296 ± 17.8	290 ± 20.0	292 ± 18.4	288 ± 17.4
Maternal bodyweight (g) day 8 (% from control)	300 ± 19.3	295 ± 20.8	294 ± 19.4	288 ± 18.1
Maternal bodyweight (g) day 9 (% from control)	304 ± 17.1	300 ± 20.4	299 ± 18.8	292 ± 15.7
Maternal bodyweight (g) day 20 (% from control)	425 ± 30.8	422 ± 27.2	415 ± 28.5	**400 ± 23.5** (-6 %)**
Maternal weight gain, days (g) 6-9 (% from control)	10 ± 7.1	11 ± 4.6	**6.0 ± 5.5* (-40)**	**1.0 ± 6.8** (-90)**
Maternal weight gain, days (g) 6-20 (% from control)	131 ± 20.2	132 ± 14.6	121 ± 16.3	**110 ± 15.9** (-16)**
Food consumption, day 0-6 (g/rat/day)	23 ± 3.0	22 ± 3.0	23 ± 1.8	23 ± 1.8
Food consumption, day 6-7 (g/rat/day) (% from control)	24 ± 3.8	21 ± 5.5	**20 ± 3.6* (-17)**	**20 ± 6.6** (-17)**
Food consumption, day 8-9 (g/rat/day) (% from control)	24 ± 3.1	24 ± 2.9	22 ± 4.1	**19 ± 3.8** (-21)**
Food consumption, days 6-20 (g/rat/day) (% from control)	26 ± 2.1	25 ± 2.3	**24 ± 2.0** (-8)**	**23 ± 1.6** (-11.5)**

* = Significantly different from the control group at 0.05 using Dunnett's test
** = Significantly different from the control group at 0.01 using Dunnett's test
Nongravid weight(s) not included in calculation of mean

Macroscopic examinations and organ weight findings (Table B 6.6.2.2)

A single female (at 550 mg/kg bw) was determined to be non-gravid and there were no macroscopic findings at any dose. Statistically significantly higher absolute and relative liver weights were noted at 550 mg/kg bw/day (+29 % and +38 % respectively) and at 225 mg/kg bw/day (12 % and 14 % respectively); liver weights in the low-dose group were unaffected by treatment with bixlozone. A clear dose-related increase in absolute and relative liver weight was observed across the doses tested. HSE considers that the magnitude of the absolute and relative liver weight increase seen at the top dose to be adverse.

Hepatocellular hypertrophy of a predominantly centrilobular distribution was evident at the mid- and high-doses, characterised by an expansion of the hepatocellular cytoplasm with pale eosinophilic cytoplasm. The severity of the findings appeared to increase with the dose tested.

Overall HSE consider that adverse liver findings were seen at the top dose.

Table B 6.6.2.2: Selected liver findings in dams from the developmental toxicity study in rats

Dosage (mg/kg/d)	Females			
	0	75	225	550
Liver weight (g)	17.8 ± 1.4	18.2 ± 1.5	**20.0 ± 1.8****	**22.9 ± 1.9****
% from control	-	+2.6	**+12**	**+29**
Liver weight relative to body weight (g / 100 net body weight)	5.37 ± 0.25	5.52 ± 0.23	**6.12 ± 0.39****	**7.42 ± 0.43****
% from control	-	+2.9	**+14**	**+38**
Histopathology - Liver [a]	25	25	25	25
Hypertrophy, hepatocellular, centrilobular	0	0	21	25
Minimal	-	-	9	0
Mild	-	-	12	7
Moderate	-	-	0	18

[a] - Number of tissues examined from each group.
** = Significantly different from the control group at 0.01 using Dunnett's test
Nongravid weights not included in calculation of the mean

GD 20 laparohysterectomy data (Table B 6.6.2.3)

Bixlozone had no effect on intrauterine growth and survival according to any of the parameters measured (post-implantation loss, live litter size, mean foetal body-weights and foetal sex ratios). Furthermore, the mean number of corpora lutea and implantation sites and the mean litter proportions of pre-implantation losses were similar across all treated and control groups.

Table B 6.6.2.3: Caesarean section data from the developmental rat study

Dose (mg/kg bw/day)	**0**	**75**	**225**	**550**
Number of females mated	25	25	25	25
Number of females with live foetuses	25	25	25	24
Non-pregnant	0	0	0	1
Mean litter size	15.6	15.6	15.4	15.8
Sex ratio (% of male)	50	54	46	50
Post-implantation loss/litter **Foetuses (dams) affected** **all early resorptions**	0.8/litter 16 (20)	0.7/litter 12 (17)	1.0/litter 25 (25)	0.8/litter 15 (19)
Pre-implantation loss	1.1/litter	0.8/litter	1.1/litter	0.8/litter
Mean foetal weight (g)	3.9	3.8	3.7	3.8

Foetal data

Morphological data (Table B 6.6.2.4)

The number of foetuses (litter) available for morphological examination were 391 (25), 390 (25), 384 (25) and 380 (24) in the control, 75, 225 and 550 mg/kg bw/d dose groups respectively. At these same respective dose-groups malformations were observed at a rate of 2 (2), 3 (2), 2(2) and 2 (2) foetuses (litter).

There were no toxicologically significant increases in malformations or variations up to the top dose. Overall there was no evidence of developmental toxicity observed at any dose tested.

Table B 6.6.2.4: Summary of malformations in the rat developmental toxicity study

Dose (mg/kg bw/day)	**0**	**75**	**225**	**550**
Total foetuses examined	**391**	**390**	**384**	**380**
Total Litters examined	**25**	**25**	**25**	**24**
External malformations				
Foetal incidence	0	1	2	1
Litter incidence	0	1	2	1
Detailed foetus (litter) incidence – External malformation				
Omphalocele	0	1 (1)	0	0
Carpal and/or tarsal flexure	0	0	1 (1)	0
Vertebral agenesis	0	0	0	1 (1)
Mandibular agnathia	0	0	1 (1)	0
Visceral (soft tissue) malformations				
Foetal incidence	2	0	0	1
Litter incidence	2	0	0	1
Detailed foetus (litter) incidence – Visceral malformation				
Right-sided aortic arch	1 (1)	0	0	0
Kidney(s)- rudimentary	1 (1)	0	0	0
Hydrocephaly	0	0	0	1 (1)
Skeletal malformations				
Foetal incidence	0	2	1	0
Litter incidence	0	1	1	0
Detailed foetus (litter) incidence – Skeletal malformation				
Only 12 pairs of ribs present	0	2 (1)	0	0
Vertebral centra anomaly	0	1 (1)	0	0
Interrupted rib(s)	0	2 (1)	0	0
Skull anomaly	0	0	1 (10	0
Total malformations				
Foetal incidence	2	3	3	2
Litter incidence	2	2	2	2

Some skeletal variations were apparent, but these were either not dose-related or only marginally increased above controls and thus considered of no toxicological significance (Table B 6.6.2.5). An increased incidence of 14th rudimentary rib and sternebrae malaligned (slight or moderate) was observed at the top-dose. However these are common variations and they did not show a dose-related response. Moreover the increases were not statistically significant compared to the controls and occurred in the presence of significant maternal toxicity. So overall they are not considered to be of toxicological significance by HSE.

Table B 6.6.2.5: Summary of variations from the rat developmental toxicity study

Dose (mg/kg bw/day)	**0**	**75**	**225**	**550**
Total foetuses examined	**391**	**390**	**384**	**380**
Total litters examined	**25**	**25**	**25**	**24**
Detailed foetus (litter) incidence - Visceral variations (absolute number)				
Renal papilla(e) not developed and/or distended ureter(s)	2 (1)	3 (3)	0	0
Liver- accessory lobule(s)	2 (1)	1 (1)	0	0
Kidney(s)- small	0	2 (1)	0	0
Haemorrhagic ring around the iris	1 (1)	1 (1)	0	0
Detailed foetus (litter) incidence - Skeletal variations (absolute number)				
Cervical centrum #1 ossified	49 (16)	66 (15)	50 (19)	51 (18)
Sternebra(e) #5 and/or #6 unossified	54 (17)	55 (21)	50 (18)	51 (15)
Hyoid unossified	6 (3)	8 (5)	3 (3)	3 (3)
14th rudimentary rib(s)	31 (8)	14 (11)	28 (13)	49 (17)
% litter incidence	7.7	3.6	7.2	13.0
Sternebra(e) malaligned (slight or moderate)	3 (2)	1 (1)	2 (2)	6 (5)
% litter incidence	0.8	0.2	0.5	1.5
7th cervical rib(s)	2 (2)	3 (3)	5 (2)	2 (2)
Sternebra(e) #1,#2,#3 and/or #4 unossified	3 (3)	5 (4)	1 (1)	3 (2)
Reduced ossification of the vertebral arches	0	3 (2)	1 (1)	3 (3)
Reduced ossification of the skull	0	6 (3)	0	0
Bent rib(s)	0	5 (3)	4 (2)	3 (2)
Pubis unossified	1 (1)	0	0	2 (2)
27 presacral vertebrae	0	0	0	1 (1)
Reduced ossification of the 13th rib(s)	2 (2)	1 (1)	1 (1)	0
Spherical enlargement of the rib(s)	0	0	1 (1)	0
Ischium unossified	0	0	0	1 (1)
Litter incidence (%) of visceral Variations	1.3	1.9	0	0
Litter incidence (%) of skeletal Variations	35.1	35.2	37.7	34.9
Total litter incidence for variations	36.1	36.4	37.7	34.9

Conclusion

In conclusion, in this guideline and GLP compliant developmental toxicity study in rats in which bixlozone was administered at 0, 75, 225 and 550 mg/kg bw/day, maternal toxicity was noted from 225 mg/kg bw/day and was characterised by a higher incidence of clinical findings with reduced food consumption and a corresponding reduction in body weight and body weight gain, which was particularly severe at the start of treatment. In addition, an adverse increase in liver weight with histopathological correlate was noted at the top dose. No evidence of developmental toxicity was observed at any dose tested.

A NOAEL for maternal toxicity of 75 mg/kg bw/day is proposed by HSE; a LOAEL of 225 mg/kg bw/day is based on clinical signs of toxicity and lower body weight and body-weight gain likely secondary to reduced food consumption. The effects on body weights were more pronounced at the start of treatment. The NOAEL for developmental toxicity is the highest dose tested of 550 mg/kg bw/day. This is consistent with the NOAELs that were proposed by the applicant.

[redacted] (2016e)

B.6.6.2.3. *Range finding pre-natal developmental toxicity study in rabbits*

A dose-range finding pre-natal developmental toxicity study has been conducted in rabbits.

Study	An Oral (Gavage) Dose Range-Finding Prenatal Developmental Toxicity Study of F9600 Technical in Rabbits
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014a)
Date performed	July – August 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105131
Guideline(s)	No claim of compliance; range-finding study
Deviations from the guideline	N/A
GLP	No
Test material	F9600 Technical Batch PL14-0049 Purity 96.0 %
Method of analysis	*Validation not required since this is a dose range-finding study.*
Study acceptable	Yes, but as supplementary study only since it is a range-finding study

Material and Methods

In a dose-range finding pre-natal developmental toxicity study bixlozone technical (batch PL14-0049, purity 96%) was administered once daily via gavage to groups of 6/dose New Zealand White rabbits throughout gestation days 7-28 at 0, 100, 350, 750 or 1000 mg/kg bw/day. The vehicle used was 0.5% [w/v] carboxymethylcellulose (CMC) in 5% Tween® 80 whilst the dosage volume for all groups was 5 mL/kg.

Results

Maternal toxicity

Clinical observations and survival

Severe maternal toxicity was noted at the top doses of 1000 and 750 mg/kg bw/day. Two females at 1000 mg/kg bw/day were found dead on GD 17 and GD 19 and a further animal at 750 mg/kg bw/day was sacrificed *in extremis* on GD 21 (body was cool to the touch). In the same group a different female aborted on GD 23 with 7 late resorptions (this female also had 2 early resorptions *in utero*). Before death these females were presented with weight loss, reduced food consumption and a corresponding decrease in defecation. The remaining females at these doses also presented with body weight losses and severely reduced food consumption and so on GD 19 all surviving females of the 750 mg/kg bw/day were sacrificed and on GD 23 all remaining females of the 1000 mg/kg bw/day group were sacrificed.

At 350 mg/kg bw/day decreased defecation was noted for three females on GD 16-21 (three occurrences each). Six incidences of rales were noted one hour post-dose in this group but occurred in a single rabbit and were not dose-related.

One female of the 100 mg/kg bw/day group aborted three dead and two live foetus on GD 28. This female had previously suffered sustained weight losses and reduced food consumption (from GD 23 until the day of abortion) and had rales on the previous day (post-dosing); necropsy revealed a thickened thymus and pericardium and white areas on the lungs, thymus and liver. There were no further deaths in this dose-group.

Overall severe maternal toxicity, leading to the death of several animals before due necropsy and the early termination of all animals in these dose groups, was observed at 1000 and 750 mg/kg bw/day. Some maternal toxicity was also noted at 300 mg/kg bw/day.

Body weight, body weight gains and food consumption

Mean body weights were lower in the 750 and 1000 mg/kg bw/day dose-groups on the day of necropsy (GD 23 and 19 respectively) by -16 % and -13 % respectively. As no females survived to the scheduled necropsy, net body weight, net body-weight gain and gravid uterine weights could not be evaluated for these dose groups.

In the lower dose groups, body weight-gain was also affected by treatment with F9600. At 350 m/kg bw/day body weight gain was lower than controls on gestation days 10-13 (-66 %) and on gestation days 13-20 (-64 %);

measurements prior to and subsequent to these were similar to controls, nevertheless when the entire gestation period was evaluated (GD7-29) body-weight gain was -27 % lower than controls (albeit without statistical significance). Similarly, at 100 mg/kg bw/d body weight gain was only affected on gestation days 10-13 and 13-20 (-49 % and -108 % respectively); body weight gain for the entire gestation period was -42 % lower than controls, but again without statistical significance. Actual mean body weight for these dose groups was similar to controls for the duration of treatment. Net body weight losses were observed at 100 and 350 mg/kg bw/day to an extent of -51.5 g and -28.4 g respectively, compared to a body weight gain of 58.1 g in the control group; net body weight and gravid uterine weights at 100 and 350 mg/kg bw/day were similar to those of the control group and any difference were slight, not statistically significant and did not show a clear dose response.

Mean food consumption in the 1000 mg/kg/day group was lower than the controls from the first day of treatment through to early termination on GD 19 to an extent ranging from -60 % to -85 % on GD 11-19. Food consumption for the 750 mg/kg bw/day was also affected by treatment with F9600 throughout gestation and until early sacrifice on GD 23; food consumption for this dose group was -60 to -86 % lower than controls on gestation days 12-23. The reductions in food consumption correlated with mean body-weight losses for these groups.

Bixlozone also affected levels of food consumption at the lower doses of 350 and 100 mg/kg bw/day. On GD 13-20 food consumption was lower than controls by -40.5 % and -33 % in the 100 and 350 mg/kg bw/day groups respectively, corresponding to lower mean body weight gains. In the preceding and succeeding gestation intervals food consumption was similar to controls; however, when the entire gestation period (GD 7-29) was evaluated, food consumption was found to be -19 % and -13 % lower than controls at 100 and 350 mg/kg bw/day respectively.

Overall body weight, body weight gain and food consumption were affected at all doses; excessive toxicity, leading to the death of several animals before due necropsy and the early termination of all animals in these dose groups, was observed at 750 and 1000 mg/kg bw/day.

Macroscopic examinations and organ weight findings

Of the females that were found dead or sacrificed *in extremis* at 750 and 1000 mg kg/bw/day all had normally developing implantation and/or early resorptions *in utero*; the only gross necropsy findings were in one female and comprised dark red areas in the stomach, one early resorption in utero and one late resorption in the vagina. The female at 100 mg/kg bw/d that had aborted three dead and two live foetuses on GD 28 (a further five live foetuses remained *in utero*) was noted with white areas on the liver, thymus and lungs and a thickened thymus and pericardium. No other internal findings were observed either in the animals that were sacrificed early or in those that survived to the scheduled necropsy; any differences were infrequent, similar to the controls and did not show a clear dose-response.

Absolute liver weights were lower than controls in the 100 and 350 mg/kg bw/day groups by a magnitude of -11 % and -7.5 % respectively, whilst in the same respective dose groups relative liver weights were lower than controls by -5 % and -4 %. The evaluation of liver weights in the higher dose groups was not possible owing to the early sacrifice of all animals in these groups.

GD 29 laparohysterectomy data

Bixlozone had no effect on the intrauterine growth or survival of the 100 and 350 mg/kg bw/day dose groups according to the parameters measured (post implantation loss, live litter size and mean foetal body weights). The number of mean corpora lutea and implantation sites were comparable across all treated and control groups; any differences from the control were slight, not statistically significant and did not occur in a dose-related manner. The evaluation of caesarean section and foetal morphology data for the 750 and 1000 mg/kg bw/day was precluded by the early termination of all animals in these dose groups.

Foetal data

The numbers of foetuses (litters) available for morphological evaluation were 46(5), 45(5), and 57(6) in the control, 100, and 350 mg/kg bw/day groups, respectively.

There were no external or visceral malformations at any dose. There were no external variations noted but visceral variations were observed in the 100 and 350 mg/kg bw/day dose groups; these comprised accessory spleen(s), major blood vessel variation (left carotid artery arose from the brachiocephalic trunk), retrocaval ureter, extra papillary muscle in the heart, and/or small gallbladder. These findings occurred infrequently or at a similar frequency to that in the concurrent control group and did not occur in a dose-related manner.

Overall there was no indication of treatment-related developmental toxicity at any doses tested.

Conclusion

In a range-finding study, administration of bixlozone to New Zealand white rabbits resulted in severe maternal toxicity at the top-doses of 1000 and 750 mg/kg bw/day that resulted in the early termination of these animals. Maternal toxicity was also evident at the lower doses of 100 and 350 mg/kg bw/day characterised by lower body-weight gain, net body-weight losses, reduced food consumption and reduced defecation. In contrast to previous studies in rats, lower absolute and relative liver weights were noted at 100 and 350 mg/kg bw/day (possibly a consequence of reduced food consumption at these doses). Owing to the early sacrifice of the rabbits at the higher doses, no liver weights or caesarean section data was evaluated at these doses. There was no indication of developmental toxicity at any dose.

Based on the results of this study, doses of 25, 75, 200 and 400 mg/kg bw/day were selected for a definitive developmental toxicity study in rabbits.

[redacted] (2014a)

B.6.6.2.4. *Pre-natal developmental toxicity study in rabbits*

The pre-natal development toxicity potential of bixlozone has been investigated in rabbits.

Study	An Oral (Gavage) Prenatal Developmental Toxicity Study of F9600 Technical in Rabbits
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2015)
Date performed	Oct 2014-Jan 2015
Test facility	[redacted]
Report reference	Study no. [redacted]-105132
Guideline(s)	OECD Guideline 414 (2001)
Deviations from the guideline	There were no deviations of significance
GLP	Yes
Test material	F9600 Technical Batch PL14-0049 Purity 96.0 %
Method of analysis	*Validation not required since the oral method of administration used was gavage.*
Study acceptable	Yes

Material and Methods

In a GLP and guideline-compliant developmental toxicity study, groups of 25 female New Zealand White rabbits were administered bixlozone with daily doses at 0, 25, 75, 200 and 400 mg/kg bw/day from gestation day (GD) 7 to GD 28 by gavage. The vehicle used was 0.5% [w/v] carboxymethylcellulose (CMC) in 5% Tween® 80 whilst the dosage volume for all groups was 5 mL/kg.

Results

Maternal toxicity

Clinical observations and survival

There were no treatment-related deaths (two deaths in the 200 mg/kg bw/day group and one death in the control group were attributed to intubation errors). With regard to clinical signs of toxicity, incidences of decreased defecation were dose-dependent and were noted in 6 females of the 400 mg/kg bw/day dose group during the second week of dose administration (Table B 6.6.2.6); other clinical signs of toxicity (hair loss, rales and red material around the mouth) occurred sporadically, were not dose-related and were also observed in the control rabbits. Overall, clinical signs of toxicity (reduced defecation) occurred at the top dose.

Body weight, body weight gains and food consumption (Table B 6.6.2.6)

Body weight-gain in the 400 mg/kg bw/day dose group was comparable with controls throughout the study; however a transient decrease in body weight-gain of -32 % was seen on GD 13-20 (albeit not statistically significant), did not affect the overall body weight gain at the end of the study, nor did it affect mean body weights throughout the treatment period; net body-weight gain, net body weights and gravid uterine weights were unaffected by treatment in this dose-group. There was no effect on body weight at any of the lower doses.

Food consumption at 400 mg/kg bw/day was lower on gestation days 13-20 (-19 %) compared to control and corresponded to the observed reductions in defecation and body weight gains at this dose during this period of gestation. The applicant has suggested that the transient lower food consumption noted during this period is not adverse owing to the lack of effect on overall mean body weights; however, since this change had an impact on the body weight gain and defecation during the same period of treatment, HSE considers the effect as adverse. There was no effect on food consumption at any other dose.

Overall a treatment-related and adverse reduction in food consumption during the second week of dosing (GD 13-20) with a corresponding reduction in body weight gain and decrease in defecation was noted at the highest dose tested of 400 mg/kg bw/day.

Table B 6.6.2.6: Body weight and food consumption from the rabbit developmental toxicity study

Dose (mg/kg bw/day)	0	25	75	200	400
Maternal bodyweight (kg)					
Day 0	3.4 ± 0.25	3.37± 0.27	3.38 ± 0.23	3.39 ± 0.28	3.37 ± 0.25
Day 7	3.48 ± 0.25	3.46± 0.26	3.44 ± 0.22	3.49 ± 0.28	3.44 ± 0.25
Day 10	3.56 ± 0.25	3.54± 0.30	3.52 ± 0.24	3.59 ± 0.30	3.50 ± 0.26
Day 13	3.60 ± 0.25	3.61± 0.32	3.57 ± 0.23	3.63 ± 0.31	3.54 ± 0.28
Day 29	3.85 ± 0.27	3.88 ± 0.30	3.82 ± 0.23	3.87 ± 0.35	3.8 ± 0.30
Maternal weight gain (g)					
Days 7-10	78 ± 64	82 ± 77	81 ± 69	99 ± 77	61 ± 90
Days 10-13	40 ± 30	64 ± 57	50 ± 63	48 ± 35	35 ± 40
Days 13-20 (% change from control)	161 ± 64	155 ± 99 (-3.7)	148 ± 90 (-8)	151 ± 63 (-6)	109 ± 99 (-32)
Days 7-29	361 ± 137	421 ± 107	383 ± 124	379 ± 184	356 ± 153
Food consumption (g/rabbit/day)					
Days 7-10	163 ± 33	160 ± 37	162 ± 37	176 ± 29	156 ± 41
Days 10-13	137 ± 26	137 ± 36	137 ± 31	145 ± 26.5	127 ± 43
Days 13-20	143 ± 28	142 ± 39 (-0.7)	138 ± 34.5 (-3.5)	144 ± 33 (+0.7)	116 ± 48.5 (-19)
Days 7-29	128 ± 19.4	131 ± 25.7	126 ± 24.6	131 ± 25.6	121 ± 30.6
Excreta					
Decreased defecation (occurrence/n° of animals)	0/0	1/1	5/2	7/2	20/6

Macroscopic examinations and organ weight findings

No unusual internal findings were observed at any dose in those animals which survived to the scheduled necropsy on gestation day 29. Any differences were slight, not statistically significant, not dose-related and occurred at similar frequencies in the control group. One female from each of the control, 25, 200 and 400 mg/kg bw/day groups were determined to be non-gravid.

In addition to one female from the control group, two females from the 200 mg/kg bw/day group were found dead on GD 12 and GD 19; all had normally developing implantations and/or early resorptions *in utero*. Macroscopic findings in these animals comprised foamy tracheal contents, dark red thoracic cavity contents and dark red areas on the lungs. All deaths occurred in close proximity to dosing and along with the macroscopic findings, indicated that the deaths were a consequence of intubation errors.

GD 29 laparohysterectomy data (Table B 6.6.2.7)

Intrauterine growth and survival were unaffected by test substance administration at dosage levels of 25, 75, 200, and 400 mg/kg bw/day. Parameters evaluated included post implantation loss, live litter size, mean foetal body weights, and foetal sex ratios. Mean numbers of corpora lutea and implantation sites and the mean litter proportions of pre-implantation loss were similar across all groups. Differences from the control group were slight and not statistically significant.

Table B 6.6.2.7: Caesarean section data from the rabbit developmental toxicity study

Dose (mg/kg bw/day)	0	25	75	200	400
Number of females mated	25	25	25	25	25
Number of females with live foetuses	23	24	25	22	24
Non-pregnant	1	1	0	1	1
Mean litter size (implantation sites)	9.6	9.1	9.6	10.0	9.2
Mean viable foetuses	9.2	8.8	8.8	9.0	8.8
Sex ratio (% of male)	48.4	51.6	50.2	51.5	51.9
Post-implantation loss/litter Total foetuses	0.3/litter 8	0.3/litter 8	0.8/litter 19	1.0/litter 22	0.5/litter 11
Pre-implantation loss Total foetuses	0.7/litter 16	0.9/litter 21	1.6/litter 40	0.8/litter 17	0.7/litter 17
Mean foetal weight (g)	41.6	42.9	41.7	41.1	41.2

Foetal data

The numbers of foetuses (litters) available for morphological evaluation were 212(23), 211(24), 221(25), 199(22), and 210(24) in the control, 25, 75, 200, and 400 mg/kg bw/day groups, respectively. Malformations were observed in 5(4), 3(2), 5(4), 3(2), and 2(2) foetuses (litters) in these same respective groups and were considered by HSE to be spontaneous in origin.

Malformations (Table B 6.6.2.8)

There were no external malformations (either treatment-related or otherwise) at any dose.

No treatment-related soft tissue malformations were observed; however several spurious soft tissue malformations were noted and are summarised in table B.6.6.2.4.3 below. These soft tissue malformations were also present at a similar frequency in the control group; furthermore none of these incidences occurred in the high-dose group; hence HSE considers that the effects were not related to treatment with bixlozone.

There were no treatment-related skeletal malformations. All of these malformations occurred at no greater incidence than the concurrent control group and did not show a clear dose-response.

Table B 6.6.2.8: Summary of the malformations found in the rabbit developmental toxicity study

Dose (mg/kg bw/day)	0	25	75	200	400
Total foetuses examined	212	211	221	199	210
Total litters examined	23	24	25	22	24
Visceral malformation					
Foetal incidence	**3**	**3**	**3**	**1**	**0**
Litter incidence	**3**	**2**	**2**	**1**	**0**
Detailed foetus (litter) incidence - Visceral malformation					
Persistent truncus arteriosus	0	1(1)	1(1)	1(1)	0
Interventricular septal defects	0	1(1)	1(1)	1 (1)	0
Lungs – lobular agenesis	2(2)	2(2)	2(1)	0	0
Vena cava – malpositioned	1(1)	0	0	0	0
Skeletal malformation					
Foetal incidence	**3**	**0**	**2**	**3**	**2**
Litter incidence	**3**	**0**	**2**	**2**	**2**
Detailed foetus (litter) incidence - Skeletal malformation					
Sternebrae fused	1 (1)	0	0	0	1
Vertebral anomaly with or without associated rib anomaly	1 (1)	0	0	2 (1)	1 (1)
Vertebral central anomaly	1(1)	0	0	0	0
Severe maligned sternebrae	0	0	2(2)	1(1)	0
Costal cartilage anomaly	0	0	1(1)	0	0
Total malformations					
Total Foetal incidence	**5**	**3**	**5**	**3**	**2**
Total Litter incidence	**4**	**2**	**4**	**2**	**2**

Variations (Table B 6.6.2.9)

No treatment-related soft tissue (visceral) variations were noted; variations that occurred in all groups included accessory spleen(s), major blood vessel variation (no brachiocephalic trunk), extra papillary muscle in the heart or only 2 papillary muscles, retrocaval ureter, absent, small, or bilobed gallbladder, small or pale spleen, pale liver, accessory liver lobule, and/or haemorrhagic ring around the iris. These visceral variations did not occur in a dose-related manner, were noted similarly in the concurrent control group, and were therefore considered to be unrelated to treatment by HSE.

No treatment-related skeletal variations were noted; findings across all groups included 13th full and/or rudimentary ribs, sternebrae (unossified, malaligned or misshapen), presacral vertebrae, extra ossification of sternebra, 7th cervical ribs or sternebra, bent hyoid arches, accessory skull bones, vertebral centra not fully ossified, reduced ossification of the skull and a hole in the xyphoid cartilage. These findings did not occur in a dose-related manner and were noted similarly in the concurrent control group; therefore, none of these were considered to be treatment-related by HSE. The incidence of sternebrae with thread-like attachment was increased (3 in 3 litters vs 0 in controls) at the top dose. However, considering the very low incidence and the isolated nature of the observation (with no clear pattern of skeletal variations), this is most likely a chance finding unrelated to treatment with bixlozone.

A distended, gas-filled stomach was observed in one foetus of the 400 mg/kg bw/day group and cystic oviducts were observed for one foetus in the control group and two foetuses in the 75 mg/kg bw/day group. These findings were not classified as either malformations or variations and hence were not included in the summary tables. In any case, they were not treatment-related (they occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not dose-related).

Table B 6.6.2.9: Summary of variations from the rabbit developmental toxicity study

Dose (mg/kg bw/day)	0	25	75	200	400
Total foetuses examined	212	211	221	199	210
Total litters examined	23	24	25	22	24
Detailed foetus (litter) incidence - Visceral variations (absolute number)					
Accessory spleen(s)	31 (14)	28 (15)	42 (18)	19 (12)	29 (14)
Heart- extra papillary muscle	9 (7)	12 (7)	7 (5)	12 (9)	7 (6)
Major blood vessel variation	7 (6)	24 (10)	8 (5)	7 (5)	15 (8)
Retrocaval ureter	1 (1)	0	4 (3)	6 (5)	1 (1)
Spleen- pale	1 (1)	2 (1)	0	0	0
Gallbladder - absent or small	0	0	0	1 (1)	0
Heart - only two papillary muscles present	1 (1)	1 (1)	0	1 (1)	3 (1)
% per litter	0.5 ± 2.32	0.4 ± 1.86	0.0 ± 0.00	0.4 ± 1.94	1.4 ± 6.80
Liver - accessory lobule(s)	1 (1)	1 (1)	0	0	2 (1)
Spleen - small	0	0	0	1 (1)	0
Liver- pale	0	0	8 (1)	1 (1)	0
Haemorrhagic ring around the iris	1 (1)	2 (2)	0	0	1 (1)
Gallbladder- bilobed	0	0	0	1 (1)	0
Detailed foetus (litter) incidence - skeletal variations (absolute number)					
13th rudimentary rib(s)	36 (17)	37 (19)	25 (16)	43 (18)	31 (19)
Sternebra(e) #5 and/or #6 unossified	24 (12)	28 (13)	16 (10)	18 (7)	25 (10)
13th full rib(s)	50 (19)	90 (22)	84 (18)	78 (16)	65 (17)
% per litter	25.7 ± 23.4	42.6 ± 24.7	37.5 ± 30.0	37.1 ± 27.5	33.5 ± 31.7
Accessory skull bone(s)	1 (1)	0	0	1 (1)	0
Extra site of ossification anterior to sternebra #1	6 (4)	3 (3)	3 (2)	5 (3)	2 (2)
Sternebrae with thread-like attachment	0	0	1 (1)	0	3 (3)
% per litter	0.0 ± 0.00	0.0 ± 0.00	0.4 ± 1.8	0.0 ± 0.00	1.4 ± 3.8
27 presacral vertebrae	4 (2)	10 (6)	14 (12)	17 (7)	8 (7)
Hyoid arch(es) bent	2 (2)	1 (1)	3 (3)	5 (4)	1 (1)
7th cervical rib(s)	1 91)	0	10 (7)	7 (4)	0
7th sternebra	1 (1)	0	1 (1)	0	0
25 presacral vertebrae	1 (1)	0	2 (2)	0	0
Sternebra(e) malaligned(slight or moderate)	2 (2)	0	5 (5)	3 (3)	0
Vertebral centra not fully ossified	0	0	1 (1)	2 (2)	0
Reduced ossification of the skull	0	0	3 (2)	0	0
Xyphoid cartilage- hole	0	0	0	1 (1)	0
Sternebra(e)- misshapen	0	0	0	1 (1)	0
Total Variations					
Litter incidence (%) of visceral Variations	**23.2**	**30.5**	**30.6**	**21.2**	**27.7**
Litter incidence (%) of skeletal Variations	**54.4**	**67.2**	**60.5**	**70.3**	**58.3**

Overall there were no indications of developmental toxicity at any doses tested.

Conclusions

In a guideline and GLP developmental toxicity study in rabbits in which bixlozone was administered by gavage at 0, 25, 75, 200 and 400 mg/kg bw/day, the signs of maternal toxicity noted were a reduction in food consumption during the second week of dosing (GD 13-20) with a corresponding reduction in body-weight gain and decrease in defecation at the highest dose tested of 400 mg/kg bw/day. The dose of 400 mg/kg bw/day thus constitutes the LOAEL for maternal toxicity in this study. There was no developmental toxicity noted up to the top dose tested. The extent of maternal toxicity observed was modest but HSE considers this adequate.

HSE proposes a NOAEL of 200 mg/kg bw/day for maternal toxicity and a NOAEL of 400 mg/kg bw/d for developmental toxicity.

The applicant proposed a NOAEL of 400 mg/kg bw/day for both maternal toxicity and developmental toxicity.

[redacted] (2015)

Table B 6.6.2.10: Summary of developmental toxicity studies with bixlozone

Method, species, test substance	Doses	NOAEL	Main effects
Rats			
Dose-range finding pre-natal developmental toxicity study, gavage **Rats**, Crl:CD(SD), females, 8/group F9600 technical, batch PL14-0049 Purity: 96 % Vehicle: 0.5 % (w/v) carboxymethylcellulose (CMC) in 5% tween® 80 [redacted] (2016d) **Supplementary**	0, 75, 225 & 550 mg/kg bw/day Dose volume: 10 mL/kg	A NOAEL was not set from this dose-range finding study	**Maternal toxicity** **675 mg/kg bw/day** One death (sacrificed on GD11) ↓ body weight ↓ body-weight gain (-36 %) ↓ net body weight (-7.5 %) ↓ net body weight gain (-45 %) ↓ food consumption (-18 %) ↑ liver weight: 64 % (relative) & 52 % (absolute) **225 mg/kg bw/day** ↑ liver weight: 17 % (absolute & relative) **75 & 25 mg/kg bw/day** No adverse effects **Developmental toxicity** **675 mg/kg bw/day** ↓ foetal body-weights
Pre-natal developmental toxicity study, gavage **Rats**, Crl:CD(SD), females, 25/group F9600 technical, batch PL14-0049 Purity: 96% Vehicle: 0.5% (w/v) carboxymethylcellulose (CMC) in 5% tween® 80 Guideline: OECD 414 (2001) Deviations: None [redacted] (2016e) **Acceptable**	0, 75, 225 & 550 mg/kg bw/day Dose volume: 10 mL/kg	**Maternal toxicity:** **75 mg/kg bw/day** Based on clinical signs, decreased body weight and body weight gain, food consumption at **LOAEL of 225 mg/kg bw/day**	**Maternal toxicity** **550 mg/kg bw/day** Clinical signs: red, yellow and/or clear material on various body surfaces Early ↓ body-weight gain: -90 % (GD 6-9)** ↓ body-weight gain: -16 % (GD 6-19)** ↓ body weight: -6 % (GD 20)** ↓ net body-weight gain: -29.5 %** ↓ net body weight: -7 %** ↓ food consumption: -11.5 % (GD 6-20)** *Organ weights* ↑ liver weight: +29 %** (absolute) & +38 %** (relative) *Histopathology* Hepatocellular hypertrophy: 7/25 (mild) & 18/25 (moderate) **225 mg/kg bw/day** Clinical signs: red, yellow and/or clear material on various body surfaces Early ↓ body-weight gain: -40 % (GD 6-9)* ↓ food consumption: -8 % (GD 6-20)* **75 mg/kg bw/day** No adverse effects

Method, species, test substance	Doses	NOAEL	Main effects
		Developmental toxicity: **> 550 mg/kg bw/d**	**Developmental toxicity** No toxicologically significant effects
Rabbits			
Dose-range finding pre-natal developmental toxicity study, gavage **Rabbits**, New Zealand White (Hra:(NZW)SPF), females, 6/group F9600 technical, batch PL14-0049 Purity: 96 % Vehicle: carboxymethylcellulose (CMC) in 5% tween® 80 [redacted] (2014a) **Supplementary**	0, 100, 350, 750 & 1000 mg/kg bw/day	A NOAEL was not set from this dose-range finding study	**Maternal toxicity** **1000 mg/kg bw/day** There were two deaths on GD 17 & 19; all remaining animals were sacrificed on GD 19 due to severe toxicity ↓ body weight -13 % ↓ food consumption -85 % **750 mg/kg bw/day** There were two deaths; all remaining animals were sacrificed on GD 23 due to severe toxicity ↓ body weight -16 % ↓ food consumption -86 % **350 mg/kg bw/day** ↓ body-weight gain -27 % (not statistically significant) ↓ net body-weight gain ↓ food consumption -13 % **100 mg/kg bw/day** ↓ body-weight gain -42 % (not statistically significant) ↓ net body-weight gain ↓ food consumption -19 % **Developmental toxicity** No treatment-related effects.
Pre-natal developmental toxicity study, gavage **Rabbits**, New Zealand White (Hra:(NZW)SPF), females, 25/group F9600 technical, batch PL14-0049 Purity: 96% Vehicle: carboxymethylcellulose (CMC) in 5% tween® 80 Guideline: OECD 414 (2001) Deviations: None GLP	0, 25, 75, 200 & 400 mg/kg bw/day	**Maternal toxicity:** **200 mg/kg bw/day** Based on decreased food consumption, body weight gain and reduced defecation at the **LOAEL of 400 mg/kg bw/day** **Developmental toxicity:** **> 400 mg/kg bw/day**	**Maternal toxicity** There were no treatment-related deaths. **400 mg/kg bw/day** ↓ defecation ↓ body-weight gain -32 % (GD 13-20) ↓ food consumption -18 % (GD 13-20) **25, 75, 200 mg/kg bw/day** No adverse effects observed **Developmental toxicity** No treatment-related effects.

Method, species, test substance	Doses	NOAEL	Main effects
[redacted] (2015) **Acceptable**			

B.6.6.3. Summary and discussion of reproductive toxicity

A full assessment of the reproductive toxicity of bixlozone has been carried out with GLP and OECD guideline compliant studies: a 2-generation reproductive toxicity study in rats in available and developmental toxicity studies have been conducted in both rats and rabbits. Range-findings reproductive / developmental studies were also conducted in the rat and rabbit and have been considered as supplementary information. Additional findings on reproductive organs from the short-term and long-term repeated dose toxicity studies are also discussed here.

Effects on Sexual Function and Fertility

The potential of bixlozone to adversely affect sexual function and fertility has been well investigated in a modern 2-generation dietary study, conducted in the rat ([redacted] (2016c)).

Bixlozone had no effect on male or female fertility or reproductive performance; gestation duration and spermatogenic endpoints were also unaffected by treatment up to the top-dose of 140 / 187 mg/kg bw/day (males / females) at which general / systemic toxicity occurred. In addition, examination of the reproductive organs did not reveal any treatment-related changes except for mononuclear cell infiltration (chronic inflammation) in the prostate which was evident in the top-dose males of both generations. In the absence of a functional effect on fertility or reproductive performance, this finding is considered of minimal toxicological significance but is accounted for by the parental NOAEL. There was also no effect on litter size, sex ratio or pup survival up to the highest dose tested in the study.

A delay in vaginal opening was seen in F1 pups at 3000 ppm (33.6 days compared with 31.7 days in controls) whilst mean body weights of these female pups at the age of attainment were unaffected by treatment with bixlozone. Therefore, it can be concluded that the delay in vaginal opening was the secondary consequence of reduced post-weaning pup female body weight development and not a specific reproductive effect of bixlozone.

Therefore, a **NOAEL for reproductive toxicity of > 3000 ppm (140 / 187 mg/kg bw/day in males / females)** can be identified from this study, based on no adverse effect on reproduction up to the highest dose tested.

In relation to general toxicity in parental animals, adequate toxicity was achieved and in line with the findings of the repeated-dose toxicity studies, this was characterised by reductions in food consumption, body weight and body weight gain and increases in relative liver weights > 15 % compared to controls accompanied by histopathological findings (hepatocellular hypertrophy) at the top dose of 140 / 187 mg/kg bw/day (lowest dose males / females) in both sexes and both generations. Adverse effects on kidneys (increase in relative kidney weights in both) were also noted at the top dose in both generations. Therefore, the top dose of ≈ 140 / 187 mg/kg bw/day constitutes the LOAEL for parental toxicity in this study. No adverse effects were observed at the lower dose of 34 / 49 mg/kg bw/day.

Consistent with the toxicity observed in the parental generations, body weights and body-weight gain of pups in the F_2 generation (but not in the F_1 generation) were affected by treatment with bixlozone at the top dose of 140 mg/kg bw/day, whilst liver weights were found to be adversely increased in male pups of the F_1 generation. Therefore, the top dose of 140 mg/kg bw/day constitutes the LOAEL for offspring toxicity in this study. No adverse effects were observed at the lower dose of 34 /49 mg/kg bw/day in both generations.

Therefore, for general **parental and offspring toxicity** a **NOAEL** of **750 ppm (34 / 49 mg/kg bw/d)** can be identified from this study.

This is consistent with the NOAELs that were proposed by the applicant.

Additional findings on reproductive organs from repeat dose toxicity studies

A slightly higher incidence of reduced epididymal sperm was seen in males from 126 mg/kg bw/day at terminal sacrifice in the 18-month mouse carcinogenicity study. No other reproductive organs were affected. No such findings were seen in the 90-day mouse study up to the top dose of 930 mg/kg bw/day. It is most likely that these mild and isolated changes occurring during the reproductive senescence of the male mouse are of minimal toxicological significance and of no relevance to the reproductive performance of the mouse.

In addition, reductions in prostate weight with associated immaturity were seen in the dog in the 90-day study from 300 mg/kg bw/day, but not up to 500 mg/kg bw/day in the 1-year study. On this basis, these prostate findings are considered to be of minimal toxicological significance and of no relevance to the reproductive performance of the dog.

Developmental toxicity

The developmental toxicity of bixlozone has been investigated in GLP and OECD guideline compliant gavage pre-natal developmental toxicity studies, conducted in the rat and rabbit. Additional information on the developmental toxicity potential of bixlozone can be extracted from the rat 2-generation study and has been taken into consideration in this summary.

In the rat developmental toxicity study ([redacted] (2016e)), maternal toxicity was noted from 225 mg/kg bw/day and was characterised by a higher incidence of clinical findings (red, yellow and/or clear material on various body surfaces), reduced food consumption and a corresponding reduction in body weight and body weight gain. Reductions in body weight gain were most marked during the first 3 days of dosing. In addition, an adverse increase in liver weight with histopathological correlate was noted at the top dose. Thus, the dose of 225 mg/kg bw/day constitutes the LOAEL for maternal toxicity. A NOAEL of 75 mg/kg bw/day for maternal toxicity is thus proposed by HSE.

No evidence of developmental toxicity was observed in the rat at any dose tested and up to doses causing clear maternal toxicity. Therefore, a NOAEL for developmental toxicity of > 550 mg/kg bw/day is proposed. The proposed NOAELs are consistent with the NOAELs that were proposed by the applicant.

In the rabbit developmental toxicity study ([redacted] (2015)), the signs of maternal toxicity noted were a reduction in food consumption during the second week of dosing (GD 13-20) with a corresponding reduction in body weight gain and decrease in defecation at the highest dose tested of 400 mg/kg bw/day. The top-dose of 400 mg/kg bw/day thus constitutes the LOAEL for maternal toxicity in this study. No adverse maternal effects were noted at lower doses. Regarding developmental findings there was no developmental toxicity noted in the rabbit up to the highest dose tested.

In conclusion HSE proposes for the rabbit study a NOAEL of 200 mg/kg bw/day for maternal toxicity and a NOAEL of 400 mg/kg bw/d for developmental toxicity. The applicant proposed a NOAEL of 400 mg/kg bw/day for both maternal toxicity and developmental toxicity.

In addition, in the rat 2-generation study, there were no effects of treatment on pup survival, sex ratio, developmental landmarks and preputial separation up to the top dose of 140 mg/kg bw/day at which parental and offspring toxicity occurred.

Overall conclusions

The overall NOAELs for reproductive toxicity are set as follows:

A **NOAEL for reproductive toxicity of 3000 ppm (140 / 187 mg/kg bw/day in males / females)** can be identified from the 2-generation reproductive toxicity study in the rat, based on no specific adverse effect on reproduction up to the highest dose tested. **A NOAEL for parental toxicity and offspring toxicity of 750 ppm (34 / 49 mg/kg bw/day in males / females)** has also been identified.

The overall NOAELs for developmental toxicity are set as follows:

No evidence of developmental toxicity was observed in the rat and rabbit up the highest doses tested at which maternal toxicity occurred. HSE proposes to set the overall **NOAEL for developmental toxicity at 400 mg/kg bw/day** based on no adverse effects observed up to the highest dose tested in the rabbit developmental study.

The overall **NOAEL for maternal toxicity is 75 mg/kg bw/day** identified from the rat developmental toxicity study.

Overall, and in accordance with Regulation GB/NI Nº 1272/2008, classification of bixlozone for reproductive and developmental toxicity is not warranted (see also aligned MCL report).

Table B 6.6.3.1: Summary of NOAELs from reproductive toxicity studies with bixlozone

Study, guideline, reference *Acceptability*	Species, doses tested	NOAEL	LOAEL	Adverse effects at LOAEL
2-generation dietary study GLP OECD Guideline 416 (2001) Deviations: None ([redacted] (2016c)) ***Acceptable***	**Rats** (Crl:CD(SD), males & females) 25/sex/group F9600 technical batch PL14-0049; purity 96 % 0, 150, 750, 3000 ppm Equivalent to doses expressed as mg/kg bw/day as in Table 6.6.1.2.1	**Parental:** **750 ppm** Equivalent to: 34/49 mg/kg bw/d in M/F **Offspring:** **750 ppm** Equivalent to 34/49 mg/kg bw/day in M/F **Reproductive:** **3000 ppm** Equivalent to 140/187 mg/kg bw/day in M/F	**Parental:** **3000 ppm** Equivalent to: 140/187 mg/kg bw/d in M/F **Offspring:** **3000 ppm** Equivalent to 140/187 mg/kg bw/day in M/F **Reproductive:** **> 3000 ppm** Equivalent to > 140/187 mg/kg bw/day in M/F	**Parental toxicity** ***F0 generation*** There were no treatment related deaths or clinical signs of toxicity. No treatment-related findings. ***F1 generation*** ↓ body-weight gains in M** & F ↓ body weights in M** & F* *Organ weights* ↑ absolute liver weights in F (+13 %**) ↑ relative liver weights: +14 %** (M) & +21 %** (F) ↑ relative kidney weights: +13 %** (M) & +10 %** (F) *Histopathology* Hepatocellular hypertrophy in F ↑ mononuclear cell infiltration (chronic inflammation) in the prostate* **Offspring toxicity** *F1 pups* ↑ relative liver weights: +18 % (M)* Delay in vaginal opening (33.6 days vs 31.7 days in controls) *F2 pups* ↓ mean body weights PND 14 (-8 %) No treatment-related findings. **Reproductive toxicity** No specific adverse effects up to top dose
Developmental gavage study GLP OECD Guideline 414 (2001)	**Rats** (Crl:CD(SD), females) 25/group F9600 Technical Batch PL14-0049	**Maternal:** 75 mg/kg bw/day	**Maternal:** 225 mg/kg bw/day	**Maternal toxicity:** Clinical signs: red, yellow and/or clear material on various body surfaces Early ↓ body-weight gain: -40 % (GD 6-9)*

Study, guideline, reference ***Acceptability***	**Species, doses tested**	**NOAEL**	**LOAEL**	**Adverse effects at LOAEL**
Deviations: None ([redacted] (2016e)) ***Acceptable***	Purity 96.0 % 0, 75, 225 & 550 mg/kg bw/day	**Developmental:** 550 mg/kg bw/day	**Developmental:** >550mg/kg bw/day	↓ food consumption: -8 % (GD 6-20)* **Developmental toxicity** No treatment-related findings.
Developmental gavage study GLP OECD Guideline 414 (2001) Deviations: None ([redacted] (2015)) ***Acceptable***	**Rabbits** (New Zealand White, females) 25/group F9600 Technical Batch PL14-0049 Purity 96.0 % 0, 25, 75, 200 & 400 mg/kg bw/day	**Maternal:** 200 mg/kg bw/day **Developmental:** 400 mg/kg bw/day	**Maternal:** 400 mg/kg bw/day **Developmental:** >400 mg/kg bw/day	**Maternal toxicity** ↓ defecation ↓ body-weight gain -32 % (GD 13-20) ↓ food consumption -18 % (GD 13-20) **Developmental toxicity** No adverse effects up to top dose

B.6.7. NEUROTOXICITY

The acute neurotoxic potential of bixlozone has been investigated in rats in a guideline-compliant acute neurotoxicity study; a dose-range finding acute neurotoxicity study was conducted beforehand and is also presented in this Section. A 90-day repeated-dose toxicity study was conducted in the rat and included dedicated neurotoxicity assessment which is presented in detail further below. For more details regarding the 90-day repeated-dose toxicity study findings please refer to Section 6.3.3 of the DAR.

B.6.7.1. Dose-range finding acute neurotoxicity study in rats

Study	An Oral (Gavage) Dose Range Finding Acute Neurotoxicity Study of F9600 Technical in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014b)
Date performed	January 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105113
Guideline(s)	Not Applicable
Deviations from the guideline	Not Applicable
GLP	No
Test material	F9600 technical Batch PL13-0385, purity 99.2 %
Method of analysis	*Validation not required since this is a dose range-finding study.*
Study acceptable	Yes, but as supplementary study only

Material and Methods

In a dose-range finding neurotoxicity study, Crl:CD Sprague Dawley rats (3/sex/group) received a single gavage dose (10 ml/kg) of bixlozone technical (batch PL13-0385, purity 99.2 %). Doses of 0, 500, 1000, 1500 and 2000 mg/kg bw were chosen according to the results of the acute oral toxicity in rats which used the same vehicle, 0.5 % (w/v) carboxymethylcellulose in 5 % Tween® 80 ([redacted] (2014a). Please refer to Section 6.2 of the DAR for further details.

Detailed clinical observations were recorded prior to dose administration and thereafter at 0.5, 2, 4 and 8 hours.

Results

There were no deaths; with regard to clinical findings, one female of the 2000 mg/kg bw dose group presented yellow material around the urogenital, ventral and abdominal areas, red material on the forelimbs, nose and mouth and decreased defecation. Another female in this group also had the same yellow material around urogenital areas. There were no other treatment related clinical findings, however sporadic findings were noted at various time points; 2 hours following treatment slightly soiled fur was noted in the females of all treated groups whilst at 4 and 8 hours post dose, in addition to the soiled fur, red deposits around the nose were observed in the 1000 and 2000 mg/kg bw males and females. Low arousal was observed in both sexes at 4 and/or 8 hours following dose administration at all dose levels including controls (hence not treatment-related). Since there was no clear dose-related response with regard to clinical findings a time to peak effect could not be determined from these results.

Table B 6.7.1.1: Parameters affected in the test substance-treated males and female rats

Finding	Dose level (mg/kg bw)	Frequency of occurrences							
		Males				Females			
		30 min	2 h	4 h	8 h	30 min	2 h	4 h	8 h
Fur appearance – slightly soiled	500	-	-	-	-	-			1/3
	1000	-	-	-	1/3	-	1/3	1/3	1/3
	1500	-	-	-	-	-		1/3	1/3
	2000	-	-	1/3	2/3	-	1/3	1/3	1/3
Red deposits – nose - present	1000	-	-	-	-	-	-	1/3	1/3
	2000	-	-	1/3	2/3	-	-	1/3	-
Arousal - low	0	-	-	1/3	1/3	-	-	1/3	-
	500	-	-	1/3	-	-	-	-	-
	1000	-	-	-	-	-	-	1/3	-
	1500	-	-	-	1/3	-	-	1/3	1/3
	2000	-	-	-	-	-	-	1/3	1/3

There was no effect on body weight, but slightly higher defecation was noted for females at 500 mg/kg bw; however, there was no dose-response observed.

Conclusion

Based on these findings, doses of 500, 1000 and 2000 mg/kg bw were selected for a definitive acute neurotoxicity study.

[redacted] (2014b)

B.6.7.2. Acute neurotoxicity study in rats

Study	An Oral (Gavage) Acute Neurotoxicity Study of F9600 Technical in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2014c)
Date performed	April 2015
Test facility	[redacted]
Report reference	Study no. [redacted]-105114
Guideline(s)	OECD N°424 (1997)
Deviations from the guideline	None
GLP	Yes
Test material	F9600 Technical Batch PL14-0049, purity 96 %
Method of analysis	*Validation not required since the oral method of administration used was gavage.*
Study acceptable	Yes

Material and Methods

In a GLP acute neurotoxicity study compliant with the OECD Guideline 424, a single oral gavage dose of bixlozone technical (batch PL14-0049, purity 96 %) was administered to groups of 10 Crl:CD Sprague Dawley rats / sex after a pre-dosing observation period of 14 days; the doses of 0, 500, 1000 or 2000 mg/kg bw were prepared in the vehicle 0.5 % (w/v) carboxymethylcellulose in 5 % Tween 80.

Animals were observed for a period of 14 days following dose administration. Functional observational battery (FOB) findings and motor activity (MA) were recorded for all animals prior to administration of the test substance and then one hour post dose (time of peak effect) on study days 0, 7 and 14. The time of peak effect was chosen based on the T_{max} of ≈ 60 minutes which was established in the pharmacokinetics and metabolism study of bixlozone in male and female Sprague-Dawley rats administered a single dose of 25 mg/kg bw ([redacted]

(2014), study Report No. FMC-R2838) since no time of peak effect could be established from the dose-range finding study.

Motor activity was assessed using a series of infrared photo beams for a duration of 60 minutes (compiled as six 10 minute intervals of 0-10 minutes, 11-20 minutes, 21-30 minutes, 31-40 minutes, 41-50 minutes, and 51-60 minutes) and is defined as a combination of fine motor skills (i.e., grooming, interruption of 1 photobeam) and ambulatory motor activity (interruption of 2 or more consecutive photobeams).

Central and peripheral nervous tissue was examined in 5 animals/sex of the control and high-dose groups.

Results

There were no deaths; clinical observations (which were recorded twice daily) revealed the presence of a yellow material around the urogenital area of four females of the high-dose group at 2000 mg/kg bw on study days 2-4 only. Although treatment-related, this observation is not considered adverse.

Body weights

Body weights were recorded weekly and there were no statistically significant differences on either body weight or body-weight gain between the treated and control groups.

FOB (Table B 6.7.2.1)

Home cage observations revealed no treatment-related findings; however, on study day 0 it was noted that 9/10 females of the 500 mg/kg bw group were sitting or standing (meaning they were not asleep or with closed eyelids) compared to 5/10 females in the control group. In the same group on study day 7 a total of 5/10 females were noted as sitting or standing and only 1/10 asleep; the changes were statistically significant compared to the control group (0/10 sitting or standing; 7/10. asleep, lying on side or curled up). These observations occurred at 500 mg/kg bw only and thus did not demonstrate any dose response relationship; furthermore, they were also not accompanied with any changes in home-cage handling and sensory parameters suggesting abnormal behaviour for this animal group. Therefore, HSE considers that this is an incidental finding not related to treatment with bixlozone.

Handling and sensory parameters were unaffected by treatment with bixlozone.

With regard to open field parameters, the time to first step was statistically significantly lower in males of the 500 mg/kg group compared to the control group; however, there was no dose-response and therefore the observation was considered a chance finding.

There were no significant effects seen on neuromuscular parameters, with the exception of a shorter mean rotarod performance which was noted in males at 2000 mg/kg bw on study day 7; this isolated finding was not repeated on days 0 or 14, therefore this effect is not considered to be related to treatment with bixlozone.

Physiological parameters were overall unaffected by test substance administration; however, several changes were noted. A statistically significantly longer catalepsy time was noted for males in the 2000 mg/kg bw group (0.6 seconds) compared with the control group (0.3 seconds) on study day 0. However, the value was similar to the values retrieved for males treated with 2000 mg/kg bw and the control group during the pre-test period (0.4 and 0.5 seconds, respectively); therefore, this difference is not considered treatment-related. Statistically significantly lower mean body temperatures were noted for females in the 1000 (36.9°C) and 2000 (37.1°C) mg/kg bw groups compared with the control group (38.0°C) at the time of peak effect (approximately 1 hour following dose administration) on study day 0. However, as no clear dose-response was seen, these slight changes are not considered treatment-related by HSE.

Overall, there were no relevant findings noted from home cage observations and examination of handling, sensory, neuromuscular and physiological parameters.

Table B 6.7.2.1: FOB findings in rats administered bixlozone by oral gavage

FOB	Parameter		F9600, dose in mg/kg bw							
			Males (n = 10)				Females (n = 10)			
			0	500	1000	2000	0	500	1000	2000
Home cage observations	Sitting or standing normally	Pre-study	6	3	6	5	7	9	6	6
		Day 0	4	3	3	2	4	6	4	5
		Day 7	3	1	3	4	0	**5***	1	4
		Day 14	4	8	8	8	8	9	10	9
	Asleep, lying on side or curled up	Pre-study	4	7	4	5	3	1	3	3
		Day 0	5	7	6	6	5	**0***	5	5
		Day 7	4	5	5	6	7	**1***	2	3
		Day 14	5	2	1	2	2	0	0	1
	Eyelid completely shut	Pre-study	4	7	4	5	3	1	3	3
		Day 0	5	6	7	6	5	**0***	5	5
		Day 7	4	5	5	6	6	1	2	3
		Day 14	4	2	1	2	-	-	-	-
Open field observations	Time to first step (s)	Pre-study	0.2 ± 0.05	0.3 ± 0.07	0.3 ± 0.05	0.3 ± 0.09	0.3 ± 0.08	0.2 ± 0.05	0.2 ± 0.05	0.2 ± 0.05
		Day 0	0.4 ± 0.05	**0.4 ± 0.07****	0.4 ± 0.03	0.4 ± 0.07	0.4 ± 0.06	0.4 ± 0.05	0.4 ± 0.04	0.4 ± 0.07
		Day 7	0.4 ± 0.07	0.4 ± 0.06	0.4 ± 0.06	0.4 ± 0.06	0.3 ± 0.04	0.3 ± 0.05	0.3 ± 0.05	0.3 ± 0.03
		Day 14	0.4 ± 0.06	0.4 ± 0.07	0.4 ± 0.06	0.4 ± 0.07	0.3 ± 0.05	0.3 ± 0.07	0.4 ± 0.05	0.4 ± 0.05
Physiological parameters	Catalepsy (s)	Pre-study	0.5 ± 0.18	0.4 ± 0.12	0.5 ± 0.19	0.4 ± 0.11	0.4 ± 0.17	0.3 ± 0.10	0.4 ± 0.18	0.6 ± 0.55
		Day 0	0.3 ± 0.09	0.5 ± 0.18	0.4 ± 0.16	**0.6 ± 0.19****	0.4 ± 0.17	0.3 ± 0.10	0.4 ± 0.18	0.6 ± 0.55
		Day 7	0.4 ± 0.45	0.4 ± 0.21	0.4 ± 0.15	0.5 ± 0.25	0.3 ± 0.16	0.4 ± 0.20	0.3 ± 0.10	0.3 ± 0.12
		Day 14	0.6 ± 0.27	0.9 ± 0.75	0.8 ± 0.77	1.5 ± 2.30	0.5 ± 0.16	0.5 ± 0.12	0.5 ± 0.12	1.7 ± 3.57
	Body temperature (degrees C)	Pre-study	37.5 ± 0.53	37.5 ± 0.47	37.3 ± 0.72	37.7 ± 0.39	37.8 ± 0.39	37.8 ± 0.97	37.8 ± 0.40	37.5 ± 0.51
		Day 0	37.6 ± 0.53	37.0 ± 1.12	37.2 ± 0.42	37.2 ± 0.46	38.0 ± 0.46	37.5 ± 0.35	**36.9 ± 1.14****	**37.1 ± 0.32****
		Day 7	37.7 ± 0.63	37.6 ± 0.37	37.8 ± 0.37	37.6 ± 0.57	37.9 ± 0.31	38.1 ± 0.56	38.0 ± 0.36	38.1 ± 0.47
		Day 14	37.8 ± 0.41	37.6 ± 0.51	37.7 ± 0.38	37.6 ± 0.56	38.4 ± 0.29	38.5 ± 0.33	38.4 ± 0.43	38.5 ± 0.39
Neuromuscular observations	Rotarod performance (s)	Pre-study	93.1 ± 46	69.6 ± 53	67.2 ± 56	68.5 ± 55	90.1 ± 43	84.1 ± 47	80.9 ± 49	75.2 ± 49
		Day 0	120 ± 0.0	96.3 ± 42	110 ± 26	84.1 ± 50	100.2 ± 42	94.2 ± 42	103.6 ± 37	62.6 ± 50
		Day 7	119 ± 3.16	114 ± 19	120 ± 0.0	**86.6 ± 44***	111.9 ± 26	103.1 ± 36	98.1 ± 46	89.3 ± 50
		Day 14	110.6 ± 30	103.4 ± 38.5	120 ± 0.0	110.7 ± 29	92.2 ± 46	101.8 ± 40	110.7 ± 29	92.1 ± 45

Significant at * $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett's test); *** $p \leq 0.001$.

Motor activity (Table B 6.7.2.2)

Lower mean total and ambulatory motor activity counts were noted on day 0 in all treated male groups during the 0-10 minute interval compared to the control group; however, the magnitude of the effect was not sufficient to affect the cumulative motor activity for the entire 60 minutes. Therefore, HSE does not consider this finding to be related to treatment with bixlozone.

In females on day 0, mean lower total and ambulatory motor activity counts were observed at the 0-10 and the 11-20 minute intervals, and in this case were of sufficient magnitude to affect the count for the entire 60 minute duration from 1000 mg/kg bw for total motor activity and from 500 mg/kg bw for ambulatory motor activity. The differences were statistically significant. However, there was no clear dose-response and the control values registered at day 0 (day of treatment) were much higher than those registered during the pre-test, with the values in the tested groups being similar to the values in the pre-treatment controls. In addition, there were no shifts in the pattern of habituation in any dose group at any time-point. Lastly, in isolation, without any effects on any other motor activity and FOB parameters, the finding is unlikely to be related to treatment. Therefore HSE concludes that the reduced motor activity (both total and ambulatory) observed in females on day 0 is not treatment-related.

Overall there were no treatment-related changes in motor activity up to the highest dose of 1000 mg/kg bw.

Table B 6.7.2.2: Cumulative total and ambulatory motor counts (0-60 minutes) in females during the pre-test and at day 0

		Dose (mg/kg bw) N = 10				HCD
		0	**500**	**1000**	**2000**	
Mean *total* motor count	Pre-test	2033	2261	2257	2491	
% difference from control		N/A	+11	+22	+22.5	
Mean *ambulatory* motor count		469	552	582	**681***	
% difference from control		N/A	+18	+24	+45	
Mean *total* motor count	Day 0	2699	2201	**1884***	**2076***	2026 [1070-2543]
% difference from control		N/A	-18.5	-30	-23	
Mean *ambulatory* motor count		736	**508***	**478***	**503***	444 [206-599]
% difference from control		N/A	-31	-35	-32	

Significant at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.
HCD: CRL:CD(SD) RAT - Date Range: Apr 2009 – Jun 2017. 406 animals, 39 studies

Pathology

There were no macroscopic findings; there were no statistically significant differences between the control and treated groups with regard to brain-weights or measurements.

Microscopically there was one incidence of focal minimal (unilateral) neuronal necrosis in the hippocampus of a single female at the highest dose of 2000 mg/kg bw. However, the study report concluded the finding to be incidental owing to the limited severity and focal unilateral nature of the finding, the lack of related histopathologic findings in other animals or in other regions of the brain in this same animal, e.g. contralateral, and the lack of any relevant clinical observations in this animal. HSE agrees with this conclusion.

Overall there were no treatment-related macroscopic or microscopic findings, or effects on brain weights or brain dimensions for males and females at any dose level tested.

Conclusion

In conclusion in this guideline and GLP acute neurotoxicity study, single oral (gavage) administration of bixlozone up to 2000 mg/kg bw resulted in no treatment-related changes in motor activity, FOB or neuropathology parameters. On this basis, HSE proposes a NOAEL for acute neurotoxicity of 2000 mg/kg bw. In addition, no clinical signs of toxicity and effects on body weights were seen up to the top dose. Therefore a NOAEL of 2000 mg/kg bw is also proposed for acute generalised toxicity.

[redacted] (2014c)

B.6.7.3. Repeated-dose neurotoxicity study in rats

One study investigating the repeated-dose toxicity of bixlozone in rats after oral administration for 90-days is available and included FOB and motor activity evaluations performed during study weeks 1, 3, 7, and 12, as well as a dedicated neurotoxicity (neuropathology) phase; the neurotoxicity findings are presented here in details. Please also refer to Section **Error! Reference source not found.** for more details on the full toxicity assessment for this study.

Study	A 90-Day Dietary Combined Toxicity and Neurotoxicity Study of F9600 in Rats
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	[redacted] (2016a)
Date performed	March-August 2014
Test facility	[redacted]
Report reference	Study no. [redacted]-105119
Guideline(s)	OECD 408 and 424
Deviations from the guideline	There were no deviations of significance
GLP	Yes (lab certified by National Authority)
Test material	F9600 Technical; batch PL14-0049 Purity 96 %,
Method of analysis	*The method is not satisfactorily validated in accordance with SANCO/3029/99 rev.4 due to the lack of explanation of the use of a non-linear calibration.* However, the method is considered sufficient for regulatory purposes.
Study acceptable	Yes

Material and Methods

Bixlozone technical was administered via the diet to Sprague Dawley rats for 90 consecutive days. Dietary concentrations were 0, 500, 2000 and 8000 ppm in males (corresponding to 0, 29, 121 and 505 mg/kg bw/day) and 0, 500, 2000 and 5000 ppm in females (corresponding to 0, 37, 150 and 351 mg/kg bw/day).

FOB and motor activity assessments were recorded for 10 animals/sex/group prior to the initiation of test diet administration and during weeks 1, 3, 7, and 12, respectively.

Six animals/sex/group were assigned to the neurotoxicity phase during study week 13, where they were deeply anesthetised and perfused *in situ*; their brain weights and dimensions (excluding olfactory bulbs) were recorded. This was followed by a neuropathological evaluation of selected tissues from the central and peripheral nervous systems performed on all animals of the control and 8000/5000 ppm groups (males and females respectively).

Results

Summary of the systemic toxicity findings observed from the toxicity groups

Treatment-related and adverse lower mean body weights and overall body weight gains (day 0-90 days) were observed at the top-dose in both sexes (8000 ppm in males and 5000 ppm in females); the effect on weight appeared to be reversible during a recovery period of 28 days. Adverse effects on food consumption and food utilisation efficiency were seen in both sexes at the top dose. In males, the decreases in food consumption were mainly seen during the first week of treatment. The effects were reversible by the end of the recovery period.

In regard to clinical-chemistry parameters, treatment-related and adverse effects on some parameters (cholesterol, proteins, calcium) indicative of potential liver toxicity were observed from 2000 ppm in both sexes. Associated histopathological findings were seen in the liver from the mid dose (2000 ppm) and findings in the thyroid were observed at the top dose (8000 / 5000 ppm in males and females respectively).

A NOAEL of 500 ppm (equivalent to 29 and 37 mg/kg bw/day in males and females respectively) has been set from this study for systemic (generalised) toxicity.

FOB (Table B 6.7.3.1)

Home cage parameters were unaffected following repeated administration to bixlozone; however several changes were noted. At week 1 a statistically significantly higher number of males were noted with faecal pellets in the mid-dose group only (2000 ppm group) compared to the control group (no defecation was seen for this group during the open field observations compared to the other male groups). The finding is not considered toxicologically relevant since it does not fit into a dose-dependent relationship for this parameter and was not repeated at the subsequent weeks. At week 12 the majority of females in the 2000 and 5000 ppm groups were noted as sitting, standing normally or alert (oriented toward observer) and the difference compared to the control group was statistically significant. Nevertheless, this isolated finding was not accompanied with a significant change in handling parameters and open field or sensory observations suggesting abnormal behaviour for these animals; the finding is thus considered to represent generalised toxicity rather than specific neurotoxicity and is covered by the NOAEL set for generalised toxicity from the study.

Handling parameters, open field and sensory observations were unaffected by treatment at all doses in both sexes.

In regard to neuromuscular parameters, the only notable change was observed at week 3 where a statistically significant shorter mean rotarod performance times were noted for males and females in the mid-dose only (2000 ppm group) when compared to their respective control groups. This isolated finding is not considered to be treatment-related since it did not fit into a dose-dependent relationship and was not repeated at the subsequent weeks.

Overall there was a higher incidence of alert females at week 12 from 2000 ppm (150 mg/kg bw/day), but, in isolation, this finding is not considered to represent a specific neurotoxic response, but generalised toxicity, already covered by the study NOAEL of 500 ppm (29/37 mg/kg bw/day in M/F see Section B.6.3).

Table B 6.7.3.1: FOB findings in rats administered bixlozone by oral gavage

FOB	Parameter		F9600, dose in ppm			
			Females (n = 10)			
			0	500	1000	2000
Home cage observations Posture	Sitting or standing normally	Pre-study	7	7	6	9
		Week 1	4	4	6	4
		Week 3	6	7	7	7
		Week 7	6	4	5	7
		Week 12	3	5	2	6
	Asleep, lying on side or curled up	Pre-study	3	3	4	1
		Week 1	2	4	4	4
		Week 3	1	0	2	2
		Week 7	1	0	1	0
		Week 12	5	4	**0***	**0***
	Sitting, head held low	Pre-study	-	-	-	-
		Week 1	-	-	-	-
		Week 3	-	-	-	-
		Week 7	-	-	-	-
		Week 12	0	0	1	0
	Alert, oriented toward observer	Pre-study	-	-	-	-
		Week 1	4	1	0	2
		Week 3	3	3	1	1
		Week 7	3	6	4	3
		Week 12	2	1	7	4
	Rearing	Pre-study	-	-	-	-
		Week 1	4	2	0	2
		Week 3	3	3	0	3
		Week 7	0	1	0	1
		Week 12	0	0	0	2

Motor activity

There were no statistically significant differences between the control and test substance-exposed groups in motor activity counts obtained from the 6 subintervals and the overall 60-minute test sessions examined at study week 1, 3, 7 and 12. No remarkable shifts in the pattern of habituation occurred in any of the exposed animals at any of the examination points (week 1, 3, 7 and 12).

*Brain weights and measurements (*Table B 6.7.3.2*)*

A slight but statistically significant increase in brain width was noted in females at the top-dose of 5000 ppm compared to the controls. At this dose level, females showed other adverse effects including lower mean body weights, body weight gains and food consumption. Since this parameter change was not accompanied with any other neuropathological findings (see histopathological finding in Section **Error! Reference source not found.** for more details), HSE considers the effect not adverse in nature and more likely related to generalised toxicity rather than neurotoxicity.

Table B 6.7.3.2: Summary of brain weights and brain measurements

	Males (n = 6)				Females (n = 6)			
	Dose (ppm)							
	0	500	2000	8000	0	500	2000	5000
Brain weight (g)	2.32 ± 0.134	2.24 ± 0.055	2.32 ± 0.078	2.31 ± 0.070	2.08 ± 0.068	2.03 ± 0.086	2.13 ± 0.071	2.12 ± 0.097
% from control		-3.4	0	-0.4		-2.4	+2.4	+1.9
Brain width (mm)	15.88 ± 0.478	15.58 ± 0.319	15.81 ± 0.451	15.79 ± 0.247	15.06 ± 0.162	15.03 ± 0.229	15.33 ± 0.314	**15.47 ± 0.321***
% from control		-1.9	-0.44	-0.57		-0.2	+1.79	**+2.72**

* Significantly different from the control group at 0.05 using Dunnett's test

Microscopic examination

No test substance-related microscopic lesions were observed in any of the central or peripheral nervous system tissues examined from 6 animals/sex in the high-dose group (8000 ppm for males and 5000 ppm for females) compared to the control group.

Conclusion

Repeated neurotoxicity was investigated as part of a guideline 90-day repeated-dose toxicity study in the rat. The FOB assessment did not show any relevant neurotoxic effect on home cage, handling, sensory or neuromuscular parameters. A higher incidence of alert females was noted at week 12 from the mid-dose of 2000 ppm (150 mg/kg bw/day), but, in isolation, this finding is not considered to represent a specific neurotoxic response. Motor activity patterns (mean ambulatory and total mean motor activity) were unaffected by treatment. There were no alterations to brain weight or length; however, a statistically significant higher group mean brain width in females at the top-dose of 5000 ppm (351 mg/kg bw/day) was noted; the finding is considered to be related to generalised toxicity rather than the expression of a specific neurotoxic effect. No test-substance related microscopic lesions or other unusual findings were noted in the central and peripheral nervous tissues.

Overall, HSE proposes a NOAEL for repeated neurotoxicity of 8000 / 5000 ppm (equating to 505 mg/kg bw/day in males and 351 mg/kg bw/day in females respectively). This is consistent with the proposal of the applicant.

[redacted] (2016a)

B.6.7.4. Delayed polyneuropathy studies

Bixlozone is not an organophosphate or a member of any other chemical class known to produce neuropathy or delayed neurotoxicity. This requirement is not triggered.

B.6.7.5. Summary of neurotoxicity

The neurotoxic potential of bixlozone has been investigated in Sprague Dawley rats in a guideline oral (gavage) acute neurotoxicity study (preceded by a range-finding study) as well as in a standard 90-day toxicity study which included a dedicated neurotoxicity phase; assessment of neurobehavioral parameters and histopathological examinations of central and peripheral nervous tissue were conducted following both acute and repeated administration of bixlozone.

In the acute neurotoxicity study, single oral (gavage) administration of bixlozone up to 2000 mg/kg bw resulted in no treatment-related changes in motor activity, FOB or neuropathology parameters. Therefore, HSE proposes a NOAEL for acute neurotoxicity of 2000 mg/kg bw. In addition, no clinical signs of toxicity and effects on body weights were seen up to the top dose. Therefore a NOAEL of 2000 mg/kg bw is also proposed for acute generalised toxicity.

Repeated neurotoxicity was investigated as part of a 90-day repeated-dose toxicity study in the rat. The FOB assessment did not show any relevant neurotoxic effect on home cage, handling, sensory or neuromuscular parameters. A higher incidence of alert females was noted at week 12 from the mid-dose of 2000 ppm (150 mg/kg bw/day), but, in isolation, this finding is not considered to represent a specific neurotoxic response. Motor activity patterns (mean ambulatory and total mean motor activity) were unaffected by treatment. There were no alterations to brain weight or length; however, a statistically significant higher group mean brain width in females at the top-dose of 5000 ppm (351 mg/kg bw/day) was noted; the finding is considered to be related to generalised toxicity rather than the expression of a specific neurotoxic effect. No test-substance related microscopic lesions or other unusual findings were noted in the central and peripheral nervous tissues.

Overall, HSE proposes a NOAEL for repeated neurotoxicity greater than the highest dietary dose tested of 8000 / 5000 ppm (equating to 505 mg/kg bw/day in males and 351 mg/kg bw/day in females respectively).

Relevant clinical findings potentially relating to neurotoxicity have also been searched in the other toxicological studies available for bixlozone, such as in acute, repeated-dose, long-term or reproductive toxicity studies conducted via the oral route in rodents (rats and mice).

Regarding the rat, the acute oral (gavage) toxicity study conducted by [redacted] (2014a) showed hypoactivity, reduced respiration (3-5 hours post administration) and decreased defecation in 2 out of 3 animals on the first

day of treatment at 2000 mg/kg bw; these signs had fully reversed by day 2. These findings are considered to represent generalised toxicity at a very high dose rather than a specific neurotoxic response. No clinical signs indicative of neurotoxicity were found in the acute dermal toxicity study conducted in rats ([redacted] (2014b)). In the acute inhalation toxicity study ([redacted] (2014c), study no. 37947), irregular respiration following exposure was observed which had fully recovered by day 3; this finding is considered to be more specifically related to the route of exposure rather than the expression of a specific neurotoxic effect. Overall, there was no clear evidence of neurotoxicity in the acute toxicity studies; however, it should be noted that no specific neurobehavioural or neuropathology investigations are generally performed in these studies. No other relevant clinical findings potentially relating to neurotoxicity were noted in the long-term or reproductive toxicity studies conducted via the oral route in the rat. In mice, no clinical findings potentially related to neurotoxicity were found in any of the studies conducted with this species.

Overall, it can be concluded that bixlozone is not neurotoxic after single or repeated administration.

Table B 6.7.5.1: Summary of neurotoxicity studies in rodents

Study and Guideline	Species/ Strain/ Groups Doses	NOAEL (mg/kg bw) [ppm]	LOAEL (mg/kg bw) [ppm]	Effects at the LOAEL
Dose-range finding acute neurotoxicity study, gavage Study no. [redacted]-105113 F9600 technical, batch PL13-0385 Purity: 99.2 % Vehicle: 0.5 % (w/v) carboxymethylcellulose in 5% Tween® 80 [redacted] 2014(b) GLP: no **Supplementary**	Rats, Crl:CD(SD), males & females, 3/sex/group 0, 500, 1000, 1500 & 2000 mg/kg bw	A NOAEL was not set from this dose-range finding study	A LOAEL was not set from this dose-range finding study	N/A
Acute neurotoxicity study, gavage Study no. [redacted]-105114 F9600 technical, batch PL14-0049 Purity: 96 % Vehicle: 0.5 % (w/v) carboxymethylcellulose in 5 % Tween® 80 Guideline: OECD 424 Deviations: none GLP [redacted] (2014c) **Acceptable**	Rats, Crl:CD(SD), males & females, 10/sex/group 0, 500, 1000 & 2000 mg/kg bw	**Neurotoxicity**: 2000 mg/kg bw **Generalised toxicity:** 2000 mg/kg bw	**Neurotoxicity**: > 2000 mg/kg bw **Generalised toxicity:** >2000 mg/kg bw	**Neurotoxicity**: No specific findings **Generalised toxicity:** No adverse findings
Repeated-dose combined toxicity and neurotoxicity study Study no. [redacted]-105119 F9600 technical, batch PL14-0049 Purity: 96 % Vehicle: 0.5 % (w/v) carboxymethylcellulose in 5 % Tween® 80 Guideline: OECD 424 Deviations: none GLP [redacted] (2016a) **Acceptable**	Rats, Crl:CD(SD), males & females, 10/sex/group Dietary doses: 0, 500, 2000 and 8000 ppm in males (0, 29, 121 and 505 mg/kg bw/day) 0, 500, 2000 and 5000 ppm in females (0, 37, 150 and 351 mg/kg bw/day).	**Neurotoxicity**: 8000/5000 ppm (505/351 mg/kg bw/day in M/F) **Generalised toxicity:** 500 ppm (29/37 mg/kg bw/day in M/F)	**Neurotoxicity**: >8000/5000 ppm (>505/351 mg/kg bw/day in M/F) **Generalised toxicity:** 2000 ppm (121/150 mg/kg bw/day in M/F)	**Neurotoxicity**: No specific effects **Generalised toxicity:** Kidney and liver effects

B.6.8. OTHER TOXICOLOGICAL STUDIES

B.6.8.1. Toxicity studies on metabolites

B.6.8.1.1. *Metabolite 2,4-dichlorobenzoic acid*

The metabolite 2,4-dichlorobenzoic acid (CAS 50-84-0, Code M190/1) is a putative major rat metabolite considered to be covered via its downstream glycine conjugate 2,4-dichlorohippuric acid, the latter being recovered in rat urine at levels > 10 % of the AD in both sexes following single low dose oral exposure.

Concentrations of the metabolite 2,4-dichlorobenzoic acid were observed above 5% in limited field dissipation studies (legacy plots prior to 10 mm of rainfall). Based on this, concentrations of the metabolite are predicted to occur in groundwater at concentrations above 0.1 µg/L. The assessment of the relevance of this metabolite according to the stepwise procedure of the EC guidance document SANCO/221/2000 –rev.10 was performed and is presented in Volume 1 Section 2.11.

Step 3, Stage 2 of the assessment requires screening for genotoxicity. In order to address this point applicant conducted the following tests: an Ames test, an in vitro mammalian cell micronucleus test in human peripheral lymphocytes and an vitro HPRT mutation test using Chinese hamster ovary cells. The tests have been evaluated by the UK and a summary of the findings are presented below.

B.6.8.1.1.1. Ames test

Study # 1	2,4-Dichlorobenzoic Acid: Bacterial Reverse Mutation Test
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	Gilby, B. (2021a)
Date performed	March 2021
Test facility	Labcorp Early Development Laboratories Ltd. U.K.
Report reference	Report no. FMC-55761
Guideline(s)	OECD 471 (2020)
Deviations from the guideline	None.
GLP	Yes
Test material	2,4-dichlorobenzoic acid; Batch P1745165 Purity 99.2% (no correction was made for purity)
Method of analysis	Not required for this test
Study acceptable	Yes.

Material and Methods

The potential of 2,4-dichlorobenzoic acid (99.2% purity) to induce gene mutations in bacteria was investigated in a GLP compliant Ames test with a plate incorporation assay (test 1) and a pre-incubation assay (test 2) using *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* strain WP2 uvrA (pKM101). A validated method of analysis for the test substance concentrations is not required for this study.

The assays were performed with and without metabolic activation (rat liver S9 from male Sprague-Dawley rats induced with phenobarbital and 5,6-benzoflavone (β-naphthaflavone)). All test items, including controls, were tested in triplicate. 2,4-Dichlorobenzoic acid was tested at concentrations of 1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate in the plate incorporation assay and at 50, 150, 500, 1500 and 5000 µg per plate for the pre-incubation assay. Dimethyl sulfoxide (DMSO) was used as the vehicle control; the following appropriate positive controls were used for the tests:

Positive, non-activation:	Sodium azide (2 µg/plate) - TA100 and TA1535 9-Aminoacridine (50 µg/plate) - TA1537 2-Nitrofluorene (2 µg/plate) - TA98 4-Nitroquinoline-1-oxide (2 µg/plate) - WP2 *uvr*A (pKM101)
Positive, activation:	2-Aminoanthracene (5 µg/plate) - TA100 and TA1535 2-Aminoanthracene (10 µg/plate) - WP2 *uvr*A (pKM101) Benzo[a]pyrene (5 µg/plate) - TA98 and TA1537

Results

Plate incorporation assay

In the plate incorporation assay there was no evidence of toxicity and no precipitate observed following exposure to the test item, up to the highest concentration recommended by the OECD guideline (limit concentration).

There was no evidence of mutagenic activity, either in the presence or absence of metabolic activation, at any concentration up to and including 5000 µg/plate. The reference mutagens used as positive controls in the test produced a distinct increase in revertant colonies and those values were within the HCD provided by the laboratory. Vehicle controls values were also within the HCD provided. Based upon these results, the concentrations of 50, 150, 500, 1500 and 5000 µg per plate were selected for use in the second test.

Table B. 6.8.1.1.: 2,4-Dichlorobenzoic acid - Plate incorporation assay

Please see below:

			Revertant Colonies (Mean / fold increase relative to vehicle)									
Metabolic Activation	**Test Item**	**Concentration (µg/plate)**	**TA98**		**TA100**		**TA1535**		**TA1537**		**WP2 *uvr*A (pKM101)**	
			Mean per plate	Fold increase	Mean per plate	Fold increase	Mean per plate	Fold increase	Mean per plate	Fold increase	Mean per plate	Fold increase
Without Activation	DMSO	100 µL/plate	44.7		138.7		30.7		12.7		231.7	
	2,4-DCBA	5	36.7	0.8	160.7	1.2	30.0	1.0	11.7	0.9	228.0	1.0
	2,4-DCBA	15	41.3	0.9	138.3	1.0	31.7	1.0	10.0	0.8	250.0	1.1
	2,4-DCBA	50	36.7	0.8	139.3	1.0	23.0	0.7	11.7	0.9	217.7	0.9
	2,4-DCBA	150	39.7	0.9	128.0	0.9	27.0	0.9	12.0	0.9	211.3	0.9
	2,4-DCBA	500	39.0	0.9	142.7	1.0	27.7	0.9	12.7	1.0	220.3	1.0
	2,4-DCBA	1500	36.7	0.8	140.7	1.0	33.0	1.1	10.0	0.8	224.0	1.0
	2,4-DCBA	5000	30.7	0.7	151.7	1.1	27.3	0.9	7.7	0.6	208.7	0.9
	2-Nitrofluorene	2	192.3	4.3	NT	NT	NT	NT	NT	NT	NT	NT
	Sodium azide	2	NT	NT	677.3	4.9	840.0	27.4	NT	NT	NT	NT
	9-Aminoacridine	50	NT	NT	NT	NT	NT	NT	113.7	9.0	NT	NT
	4-Nitroquinoline-1-oxide	2	NT	NT	NT	NT	NT	NT	NT	NT	2131.3	9.2
With Activation	DMSO	100 µL/plate	55.0		161.7		22.7		14.0		229.3	
	2,4-DCBA	5	51.0	0.9	141.3	0.9	19.7	0.9	12.0	0.9	207.3	0.9
	2,4-DCBA	15	56.7	1.0	154.0	1.0	20.7	0.9	17.7	1.3	217.3	1.3
	2,4-DCBA	50	55.3	1.0	156.3	1.0	16.0	0.7	14.3	1.0	230.0	1.0
	2,4-DCBA	150	44.0	0.8	159.0	1.0	19.0	0.8	18.3	1.3	225.7	1.3
	2,4-DCBA	500	45.0	0.8	170.0	1.1	19.3	0.9	20.7	1.5	223.7	1.5
	2,4-DCBA	1500	46.3	0.8	177.0	1.1	21.0	0.9	13.0	0.9	228.7	0.9
	2,4-DCBA	5000	37.3	0.7	208.3	1.3	14.0	0.6	8.7	0.6	195.0	0.6
	Benzo(a)pyrene	5	273.7	5.0	NT	NT	NT	NT	69.7	5.0	NT	NT
	2-Aminoanthracene	5	NT	NT	1730.7	10.7	450.3	19.9	NT	NT	NT	NT
	2-Aminoanthracene	10	NT	NT	NT	NT	NT	NT	NT	NT	959.3	3.7

NT Not tested

Pre-incubation assay (test 2)

In the pre-incubation assay there was no evidence of toxicity and no precipitate observed in strains TA100, TA1535 and WP2 uvrA (pKM101) following exposure to the test item, up to the highest concentration tested (5000 µg/plate). There was no evidence of mutagenic activity, either in the presence or absence of metabolic activation, at any concentration in these strains.

Cytotoxicity (reduction in revertant colony numbers compared to controls) was observed at the top concentration in strains TA98 and from 1500 µg/plate in strain TA1537 in the absence of S9 mix, and in strain TA1537 in the presence of S9 mix. There was no indication of a biological or dose-dependent increase in the number of revertants in these strains, either in the presence or absence of metabolic activation, at any concentration up to cytotoxic concentrations. The reference mutagens used as positive controls in the test produced a distinct increase in revertant colonies and those values were within the HCD provided by the laboratory. Vehicle controls values were also within the HCD provided.

Table B. 6.8.1.1.1.1: Experiment 2 Pre-incubation assay

Metabolic Activation	Test Item	Concentration (µg/plate)	TA98		TA100		TA1535		TA1537		WP2 *uvr*A (pKM101)	
			Mean per plate	Fold increase	Mean per plate	Fold increase	Mean per plate	Fold increase	Mean per plate	Fold increase	Mean per plate	Fold increase
Without Activation	DMSO	100 µL/plate	42.7		184.7		34.0		17.3		214.0	
	2,4-DCBA	50	49.0	1.1	164.3	0.9	25.7	0.8	19.0	1.1	223.0	1.0
	2,4-DCBA	150	32.0	0.8	163.0	0.9	25.0	0.7	19.0	1.1	198.7	0.9
	2,4-DCBA	500	32.7	0.8	155.0	0.8	22.7	0.7	14.3	0.8	180.3	0.8
	2,4-DCBA	1500	29.3	0.7	156.7	0.8	24.0	0.7	*11.7*	*0.7*	153.7	0.7
	2,4-DCBA	5000	*22.7*	*0.5*	189.0	1.0	25.7	0.8	*4.7*	*0.3*	143.0	0.7
	2-Nitrofluorene	2	231.3	5.4	NT	NT	NT	NT	NT	NT	NT	NT
	Sodium azide	2	NT	NT	819.7	4.4	931.3	27.4	NT	NT	NT	NT
	9-Aminoacridine	50	NT	NT	NT	NT	NT	NT	147.7	8.5	NT	NT
	4-Nitroquinoline-1-oxide	2	NT	NT	NT	NT	NT	NT	NT	NT	2781.0	13.0
With Activation	DMSO	100 µL/plate	48.3		184.3		22.7		23.3		261.3	
	2,4-DCBA	50	49.3	1.0	151.3	0.8	19.3	0.9	16.0	0.7	231.7	0.9
	2,4-DCBA	150	46.0	1.0	149.0	0.8	16.3	0.7	19.7	0.8	239.7	0.9
	2,4-DCBA	500	46.3	1.0	168.0	0.9	17.3	0.8	20.3	0.9	239.3	0.9
	2,4-DCBA	1500	49.3	1.0	210.0	1.1	18.0	0.8	17.7	0.8	225.0	0.9
	2,4-DCBA	5000	37.3	0.8	222.3	1.2	14.7	0.6	*12.0*	*0.5*	156.7	0.6
	Benzo(a)pyrene	5	253.7	5.2	NT	NT	NT	NT	77.0	3.3	NT	NT
	2-Aminoanthracene	5	NT	NT	2764.7	15.0	508.7	22.4	NT	NT	NT	NT
	2-Aminoanthracene	10	NT	NT	NT	NT	NT	NT	NT	NT	1276.3	4.9

NT Not tested
In italics: cytotoxicity (reduction in revertant colony numbers compared to controls)

Conclusion

The results from this GLP and OECD guideline Ames study shows that 2,4-dichlorobenzoic acid does not have the ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system up to the concentration limit of the test (5000 µg per plate) or up to cytotoxicity concentrations.

Overall, there was no evidence of a mutagenic potential of 2,4-dichlorobenzoic acid in this study.

Gilby, B. (2021a)

B.6.8.1.1.2. In Vitro Mammalian Cell Micronucleus Test in Human Peripheral Lymphocytes

Study # 1	2,4-Dichlorobenzoic Acid: In Vitro Mammalian Cell Micronucleus Test in Human Peripheral Lymphocytes
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	Gilby, B. (2021b)
Date performed	March 2021
Test facility	Covance Laboratories Limited U.K.
Report reference	Report no. FMC-55762
Guideline(s)	OECD 487 (2016)
Deviations from the guideline	Yes, but it did not impact on the validity of the study for regulatory purposes. In this study the total DMSO volume in the 20-hour treatments samples was 1.5% w/w (1% w/w vehicle or dose formulation and 0.5% w/w cytoB) and no untreated control cultures were included. The OECD 487 (2016) guideline states that "If cytoB is dissolved in DMSO, the total amount of organic solvent used for both the test chemical and cytoB should not exceed 1% (v/v); otherwise, untreated controls should be used to ensure that the percentage of organic solvent has no adverse effect". Since previous studies in this laboratory did not indicate any deleterious effects where DMSO was dosed at 1.5% (v/v) the inclusion of untreated control cultures was not deemed to be necessary. In addition, the vehicle controls of the 20-hour treatments gave comparable results both in terms of CBPI values and micronucleus frequencies to those in the laboratories historical control database. The justification provided by the applicant is considered acceptable by HSE. Thus, the use of 1.5% (v/v) DMSO was considered not to have any adverse effects in this study, and the study is valid for regulatory purposes.
GLP	Yes
Test material	2,4-dichlorobenzoic acid; Batch P1745165 Purity 99.2% (no correction was made for purity)
Method of analysis	Not required for this test
Study acceptable	Yes.

Material and Methods

Materials and Methods

The metabolite 2,4-dichlorobenzoic acid was investigated for its potential to induce micronuclei in human lymphocytes in vitro in the presence or absence of metabolic activation (phenobarbital/β-naphthoflavone induced rat liver S9) in a GLP and OECD test guideline compliant study.

The micronucleus *in vitro* assay consisted of a preliminary toxicity test and a main micronucleus test. The lymphocytes were exposed to the vehicle, positive control or 2,4-dichlorobenzoic acid in duplicate cultures of cells for 3 hours (with and without metabolic activation) or 20 hours (without metabolic activation) with the following concentrations:

Test substance (2,4-DCBA) concentrations used in the preliminary toxicity test

All treatments (-/+S9 mix (3 hours) and -S9 mix (20 hours)	3.73, 7.46, 14.92, 29.85, 59.69, 119.38, 238.76, 477.53, 995.05 and 1910.1* µg/mL in single cultures.

*1910.1 µg/mL is equivalent to 10 mM, the standard limit concentration within this test system as recommended in the current OECD 487 (2016) guideline

In the main experiment, three concentrations from those tested were evaluated for mutagenicity :

Test substance (2,4-DCBA) concentrations used in the main micronucleus test (cultures analysed for micronucleus frequency are underlined)

-S9 3-hour exposure group	666.01, 740, 822.24, 913.59, 1015.11, 1127.89, 1253.22, 1392.46, 1547.18, 1719.09 and 1910.1 µg/mL in duplicate in the absence of S9 activation.
+S9 3-hour exposure group	666.01, 740, 822.24, 913.59, 1015.11, 1127.89, 1253.22, 1392.46, 1547.18, 1719.09 and 1910.1 µg/mL in duplicate in the presence of S9 activation.
-S9 20-hour exposure group	39.33, 393.27, 436.97, 485.52, 539.47, 599.41, 666.01, 740, 822.24 and 913.59 µg/mL in duplicate in the absence of S9 activation.

In both experiments the solvent DMSO (1.0 % in culture medium) served as a negative control; mitomycin C (3-hr) and colchicine (20-hr) were used as positive controls in the absence of S9 mix, and cyclophosphamide as a positive control in the presence of S9 mix, to demonstrate appropriately the validity of the experiment. Treatment was started after a 48-hour stimulation period triggered by phytohemagglutinin (PHA). After any necessary treatment with the S9 mix with subsequent washing and recovery phases, cytochalasin B (6.0 µg/mL) was added to the cultures to arrest the cell cycle. The cultures were then prepared after another 20 hours.

The CBPI was determined in 500 cells per culture. Any cytotoxicity effects of the test item on the cells was expressed as reduction in the CBPI compared with vehicle control values (cytostasis) or cell death. The number of binucleate cells per culture scored for micronuclei on coded slides was 1000.

Results

Osmolarity and pH:

A slight but dose-dependent decrease in pH was observed up to the highest concentration tested (1910.1 µg/mL) when compared with the concurrent vehicle controls; the shift is, at less than 1.0 in unit and ranged between 6.76 and 7.08, not considered to affect the experiment. No relevant changes in osmolarity was noted.

Preliminary toxicity test

Precipitation was observed at the top concentration following 3 hour treatments in the absence and presence of S9 mix and no precipitation was observed in the 20-hour treatment in the absence of S9 mix.

After 3-hour treatment in the absence of S9-mix, there was no cytotoxicity (0% cytostasis) observed at concentrations up to 995.05 µg/mL. However, at the higher concentration (maximum tested) excessive cytotoxicity was observed.

After 3-hour treatment in the presence of S9-mix, cytostasis was observed in most of the samples with the test item, reaching 59.2% at the maximum concentration tested (1910.1 µg/mL).

After 20-hour treatment in the absence of S9-mix, cytostasis was observed with the test item from 29.85 µg/mL and was excessive (87.1%) at 995.05 µg/mL.

Main micronucleus test

After 3-hour treatment in the absence and presence of S9-mix, there was precipitation observed at 1547.18 µg/mL and above. Cytostasis was observed in most of the samples with the test item, which was significant at 1547.18 µg/mL (53.5% and 22.0 % cytostasis in absence and presence of S9-mix respectively). Thus, the concentrations of 822.24, 1392.46 and 1547.18 were selected for micronucleus analysis.

After 20-hour treatment in the absence of S9-mix, there was no precipitation observed up to the highest concentration tested. Cytostasis was observed in all the samples tested, reaching 50.2% compared with vehicle

control values at 599.41 µg/mL; overt cytotoxicity was observed above that concentration. Thus, the concentrations of 39.33, 485.52 and 599.41 were selected for micronucleus analysis.

In all experiments, there was no biologically relevant or dose-dependent increase in the numbers of micronucleated cells (binucleated cells containing micronuclei per 1000 cells) after treatment with the test item after 3 or 20 hours, either in the presence and absence of metabolic activation, up to the highest analysable concentrations tested. The mean micronucleus frequencies were all within the laboratory HCD 95% confidence limits for the vehicle controls.

Validity

All solvent negative control values were within the mean + SD values of the HCD expect for the 20 hours treatment without S9-mix, and all values were within the range of the laboratory HCD (95% confidence limits). The positive controls showed distinct and statistically significant increases in cells with micronuclei across the experiments, so demonstrating the sensitivity of the test system. All positive control values were within the mean + SD values of the HCD expect for the 3 hours treatment with S9-mix, and all values were within the range of the laboratory HCD for positive controls. Thus, the results verified the validity of the study.

Table B. 6.8.1.1.2.1: Summary of cytotoxicity and clastogenicity/aneugenicity data – Main micronucleus test

Treatment Condition	Test item concentration (µg/mL)	Cytostasis (%)	Binucleated cells containing micronuclei per 1000 cells[a]			
			Mean	*p*-value[b]	Trend test *p*-value[c]	Laboratory HCD[f] N studies (cultures) Mean ± SD *95% confidence limits*
3-hour –S9	Vehicle[d]	0.0	4.5			N = 68 (272) Mean 6.06 ± 2.4 *CL: 1.8 – 11.4*
	2,4-DCBA (822.24)	2.7	5.0	1.000		
	2,4-DCBA (1392.46)	22.9	3.5	0.654	0.549	
	2,4-DCBA (1547.18)[e]	53.5	2.5	0.213	0.119	
	MMC (0.3)	32.0	28.0	**<0.001*** **		N = 68 (136) Mean 39.0 ± 12.5 *CL: 24.6 – 64.0*
	COL (0.07)	47.2	19.5	**<0.001*** **		N = 68 (136) Mean 25.6 ± 7.5 *CL: 10.6 – 40.6*
3-hour +S9	Vehicle[d]	0.0	4.5			N = 67 (268) Mean 6.5 ± 2.2 *CL: 2.0 – 11.0*
	2,4-DCBA (822.24)	4.5	4.5	0.935		
	2,4-DCBA (1392.46)	16.8	4.0	0.801	0.672	
	2,4-DCBA (1547.18)[e]	22.0	4.0	0.801	0.579	
	CPA (10)	21.4	16.5	**<0.001*** **		N = 67 (134) Mean 22.2 ± 5.7 *CL: 10.9 – 33.6*
20-hour -S9	Vehicle[d]	0.0	3.3			N = 67 (267) Mean 6.7 ± 2.3 *CL: 2.0 –11.4*
	2,4-DCBA (39.33)	2.5	3.0	1.000		
	2,4-DCBA (485.52)	27.8	3.0	1.000	0.820	
	2,4-DCBA (599.41)	50.2	3.5	0.851	0.691	
	MMC (0.1)	31.9	19.0	**<0.001*** **		N = 67 (134) Mean 25.4 ± 7.3 *CL: 16.2 – 39.9*
	COL (0.02)	41.8	15.5	**<0.001*** **		N = 67 (134) Mean 17.0 ± 4.0

						CL: 9.0 – 25.1

a. The number of micronucleated cells determined in a sample of 2000 binucleate cells (4000 for vehicle)
b. *p*-values are for comparisons to control using Williams' test for 2,4-DCBA and the *t*-test otherwise
c. Trend test *p*-values are for the linear contrast including the control group and lower concentrations of the same test item
d. Vehicle control = DMSO 1% (v/v)
e. Precipitate observed by eye at the end of treatment
f. Data range: Feb 2019 – Feb 2021
*** $p<0.001$
CBPI: Cytokinesis block proliferative index
MMC: Mitomycin C
COL: Colchicine
CPA: Cyclophosphamide
HCD: Historical control data
SD: Standard deviation
CL: Concentration limits

Conclusion

In conclusion, under the conditions of this OECD test guideline and GLP compliant study, the test item 2,4-dichlorobenzoic acid did not induce micronuclei in the absence of metabolic activation up to cytotoxic concentrations, as determined by this *in vitro* micronucleus test in human lymphocytes. Therefore, 2,4-dichlorobenzoic acid is considered not to be clastogenic and aneugenic, in this *in vitro* micronucleus test when tested up to cytotoxic concentrations.

Gilby, B. (2021b)

B.6.8.1.1.3. In Vitro *Hprt* Mutation Test Using Chinese Hamster Ovary Cells

Study # 1	2,4-Dichlorobenzoic Acid: In Vitro Hprt Mutation Test Using Chinese Hamster Ovary Cells
Evaluation status:	**New data, submitted for purpose of first approval in GB**
Reference	Gilby, B. (2021c)
Date performed	April 2021
Test facility	Labcorp Early Development Laboratories Ltd. U.K.
Report reference	Report no. FMC-55811
Guideline(s)	OECD 476 (2016)
Deviations from the guideline	None.
GLP	Yes
Test material	2,4-dichlorobenzoic acid; Batch P1745165 Purity 99.2% (no correction was made for purity)
Method of analysis	Not required for this test
Study acceptable	Yes.

Material and Methods

The test item 2,4-dichlorobenzoic acid was tested for its potential to induce gene mutations at the *Hprt* locus *in vitro* in Chinese hamster ovary (CHO-K1) cells in both the presence and absence of metabolic activation, using S9 mix from rat livers induced with phenobarbital and 5,6-benzoflavone.

In this study, concurrent solvent controls (culture medium with 1 % DMSO) were used as the negative control. Appropriate reference mutagens were used as positive controls to demonstrate the validity of the experiment:

Positive control mutagens tested

Condition	Mutagen	Solvent	Conc.
Without metabolic activation	Ethylmethanesulphonate (EMS)	DMSO	250 µg/mL
With metabolic activation	3-Methylcholanthrene (3MC)	DMSO	5 µg/mL

The assay was performed in duplicate cultures where the cells were incubated for 3 hours (either with or without metabolic activation).

To evaluate cytotoxicity of the test item and determine concentrations to be used in the main experiment, a pre-experiment was carried out. The pre-test was performed with concentrations of the test item ranging between 14.92 µg/mL and 1910.1 µg/mL, with an exposure time of 3 hours in the presence and absence of metabolic activation. The pH and osmolarity were also measured in this pre-experiment.

Test substance (2,4-DCBA) concentrations used in the preliminary toxicity test:

All treatments (-/+S9 mix (3 hours)	14.92, 29.85, 59.69, 119.38, 238.76, 477.53, 955.05 and 1910.1 µg/mL

Based on the results of this pre-experiment, concentrations of up to 1910.9 µg/mL (equivalent to 10 mM)– the highest test concentration recommended by OECD 476 - were used in the main experiment.

Each concentration and the controls were tested in duplicate. In the main experiment, the test item was tested at the following concentrations (cultures assessed for determination of the mutant phenotype are underlined):

-S9 mix (3 hours)	50, 500, 1000, 1400 and 1910.1 µg/mL in duplicate in the absence of S9 activation.
-S9 mix (3 hours) Additional test	458.62, 655.16, 935.95, 1337.07 and 1910.1 µg/mL in duplicate in the absence of S9 activation.
+S9 mix (3 hours)	50, 500, 1000, 1200, 1400, 1600, 1800 and 1910.1 µg/mL in duplicate in the presence of S9 activation.

The exposure time for the main experiment, both with and without metabolic activation, was 3 hours. After the incubation period, the treatment media were replaced with fresh medium and cells were trypsinised and then incubated for 7 days to allow for expression of mutant cells. After these 7 days, the cells were incubated in selective medium containing 6-TG (final concentration of 10 µg/mL) for approximately 7 days, before being stained in a methanol:Giemsa solution (4:1 v/v) and counted. Two additional flasks were seeded per experimental point with approx. 500 cells each to determine the relative survival (RS) as a measure of test item induced cytotoxicity. The mean mutant frequency (MF) is calculated based on the number of mutant colonies corrected by the cloning efficiency at the time of mutant selection.

Results

Pre-experiment testing cytotoxicity, pH and osmolarity:

There was no visual precipitation observed after 3 hours treatment, with and without metabolic activation. There was no relevant change in pH and osmolarity of the medium, up to the highest tested concentration (1910.1 µg/mL) compared with the concurrent vehicle controls.

No significant cytotoxic effect, indicated by a relative cloning efficiency (RS) of <50 %, was observed after 3 hours treatment without metabolic activation, up to the highest tested concentration and compared to the vehicle controls. After 3 hours treatment with metabolic activation, no significant cytotoxic effect was observed up to the concentration of 955.05 µg/mL; excessive cytotoxicity (4% RS) was seen at the highest concentration tested.

Concentrations for the main test were based upon these data.

Main experiment

3-hour treatment in the absence of metabolic activation

After 3-hour treatment in the absence of metabolic activation, there were no precipitation, no significant cytotoxicity and no concentration-related response or statistically significant increase (tests for linear trend and non-linearity) in mean MF numbers/10^6 cells, up to the maximum concentration of 1910.9 µg/mL compared to the vehicle controls. The positive controls, EMS showed a distinct and statistically significant increase in induced mutant colonies, demonstrating the sensitivity of the test system, and both the negative and positive controls fulfilled the acceptability criteria of the laboratory and the OECD guideline, demonstrating the validity of the experiment.

The mean mutant frequencies for the test item treated cultures at 50, 500 and 1400 µg/mL were above the HCD 95% confidence limits. All the other results, including those of the vehicle and positive controls, were within the HCD 95% confidence limits. Thus, the results did not fulfill the criteria for a clearly negative result according to the OECD guideline, and an additional test was conducted accordingly.

Additional 3-hour treatment in the absence of metabolic activation

After 3-hour treatment in the absence of metabolic activation, there were no precipitation, no significant cytotoxicity and no concentration-related response or statistically significant increase (tests for linear trend and non-linearity) in mean mutant colony numbers/10^6 cells, up to the maximum concentration of 1910.9 µg/mL compared to the vehicle controls. The positive controls, EMS showed a distinct and statistically significant increase in induced mutant colonies, demonstrating the sensitivity of the test system, and both the negative and positive controls fulfilled the acceptability criteria of the laboratory and the OECD guideline, demonstrating the validity of the experiment.

The mean mutant frequency for the vehicle control cultures was slightly above the laboratory HCD 95% confidence limits (13.63 vs. 13.5) but is acceptable for inclusion in the historical control distribution according to the OECD guideline recommendations (paragraph 33 of the guideline). The mean mutant frequency for the test item treated cultures at 935.95 µg/mL was also above the HCD 95% confidence limits of the vehicle control (13.99 vs. 13.5) however, the value is within the HCD range (5.6 – 14.5) and is comparable to the value of the vehicle control in this experiment (Mean MF = 13.99 at 935.95 µg/mL vs MF = 13.63 in control). All the remaining test item treated cultures had mean mutant frequencies within the HCD 95% confidence limits.

Conclusion on the two independent 3-hour treatments in the absence of metabolic activation

In the two independent tests in the absence of S9 mix, the test item did not cause any statistically significant increases in mean MF compared to the controls and showed no evidence of a concentration-related increase. Both negative and positive controls fulfilled the acceptability criteria of the laboratory and the OECD guideline. In the second experiment, the mean MFs which were above the HCD 95% confidence limits were only slightly exceeding those limits, they remained within the HCD range and were comparable to the control value. Lastly, all the MFs were within the expected spontaneous mutant frequency range of between 5 and 20 x 10^{-6} mutants as described in the OECD 476 (2016) guideline (paragraph 26 of the guideline).

Overall, the results in the absence of S9 mix were considered negative.

3-hour treatment in presence of metabolic activation

There were no precipitation observed at the end of treatment, but the test item caused excessive toxicity at 1800 µg/mL (mean RS to 20%). There was no concentration-related response or statistically significant increase (tests for linear trend and non-linearity) in mean mutant colony numbers/10^6 cells, up to the maximum (cytotoxic) concentration of 1800 µg/mL compared to the vehicle controls. The 95 % confidence interval of the laboratory HCD range was not exceeded in any of the treated samples.

The positive control, 3MC, showed a distinct and statistically significant increase in induced mutant colonies, demonstrating the sensitivity of the test system, and both the negative and positive controls fulfilled the acceptability criteria of the laboratory and the OECD guideline, demonstrating the validity of the experiment.

Overall, the results in presence of S9 mix were considered negative.

Table B. 6.8.1.1.3.1: Summary of cytotoxicity and Hprt mutation test data

Test Item	Concentration (µg/mL)	3-hour Treatment -S9 mix		Additional 3-hour Treatment -S9 mix		Laboratory HCD[b] N studies Mean ± SD *95% confidence limits*	3-hour Treatment +S9 mix		Laboratory HCD[b] N studies Mean ± SD *95% confidence limits*
		Mean RS (%)	Mean MF[a]	Mean RS (%)	Mean MF[a]		Mean RS (%)	Mean MF[a]	
DMSO	0	100	12.39	100	13.63	N = 26 Mean 8.6 ± 2.5 *CL:3.6 – 13.5*	100	8.06	N = 29 Mean 9.0 ± 3.4 *CL: 2.2 – 15.9*
2,4-DCBA	50	103	17.66	-	-		93	NP	
2,4-DCBA	458.62	-	-	101	12.05		-	-	
2,4-DCBA	500	92	17.51	-	-		88	NP	
2,4-DCBA	655.16	-	-	108	13.49		-	-	
2,4-DCBA	936.95	-	-	119	13.99		-	-	
2,4-DCBA	1000	106	11.64	-	-		92	12.26	
2,4-DCBA	1200	-	-	-	-		91	10.37	
2,4-DCBA	1337.07	-	-	110	11.02		-	-	
2,4-DCBA	1400	100	14.60	-	-		69	11.22	
2,4-DCBA	1600	-	-	-	-		50	6.64	
2,4-DCBA	1800	-	-	-	-		20	7.36	
2,4-DCBA	1910.1	102	11.61	99	13.28		3	NP	
EMS	250	107	**85.20*****	100	**76.82*****	N = 26 Mean 77.7 ± 23.4 CL: 30.9 – 124.5	-	-	
3MC	5	-	-	-	-		89	**125.54*****	N = 29 Mean 76.7 ± 22.0 *CL: 32.7 – 120.6*

a. Mutant frequencies expressed per 10^6 viable cells
b. 18 July 2019 to 31 March 2021

Grey cells: not tested
RS: Relative Survival
MF: Mutant Frequency
NT: Not tested
NP: Not plated for assessment of mutant frequency
HCD: Historical control data
EMS: Ethyl methanesulphonate
3MC: 3-methylcholanthrene
*** $p<0.001$; all other cultures $p\geq0.05$. Treated groups were compared to the vehicle control using one-tailed Dunnett's tests for an increase and the positive control was compared to the vehicle control using a one-tailed *t*-test for an increase

Conclusion

Under the conditions of this GLP and OECD test guideline compliant study, the bixlozone metabolite 2,4-dichlorobenzoic acid did not induce gene mutations at the *Hprt* locus in CHO-K1cells in vitro when tested up to cytotoxicity or the limit concentration for this assay. Therefore, in accordance with this *Hprt* assay, 2,4-dichlorobenzoic acid is considered not to be mutagenic.

Gilby, B. (2021c)

B.6.8.1.1.4. Summary and conclusion of genotoxicity testing of the metabolite 2,4-dichlorobenzoic acid

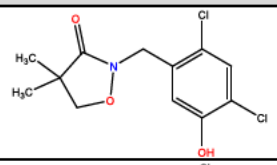
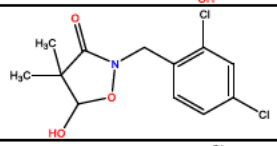
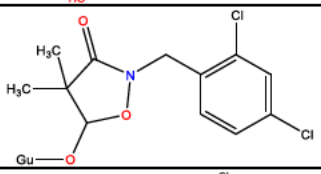
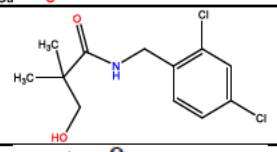
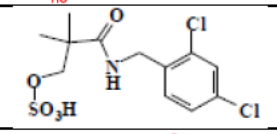
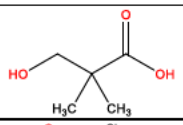
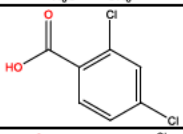
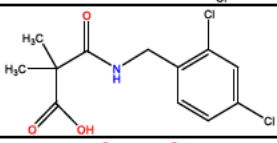
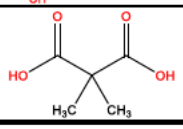
Test system and Acceptability	Concentration/ dose levels	Purity (%)	Results	Reference
***In vitro* studies**				
Ames Tests (Reverse mutation assay) *S. typhimurium* strains (TA 98, TA 100, TA 1535, TA 1537) *Escherichia coli* strain WP2 uvrA (pKM101) +/– S9 *Acceptable modern study*	plate incorporation assay (test 1): 1.5 - 5000 µg/plate pre-incubation assay (test 2): 50-5000 µg/plate Batch : P1745165	99.2	Negative	Gilby, B., 2021 (FMC-55761)
In vitro micronucleus tests in human lymphocytes +/– S9 *Acceptable modern study*	Preliminary toxicity test: 3.73 – 1910.1 µg/mL Main micronucleus test: 39.33 – 1910.1 µg/mL Batch : P1745165	99.2	Negative	Gilby, B., 2021 (FMC-55762)
In Vitro Hprt Mutation Test Using Chinese Hamster Ovary Cells +/– S9 *Acceptable modern study*	Preliminary toxicity test: 14.92 – 1910.1 µg/mL Main Experiment: 50 – 1910.1 µg/mL Batch : P1745165	99.2	Negative	Gilby, B., 2021 (FMC-55761)

The genotoxic potential of the bixlozone metabolite 2,4-dichlorobenzoic acid was investigated in a standard battery of *in vitro* tests. The metabolite was tested in an *in vitro* Salmonella typhimurium reverse mutation (Ames) test, an *in vitro* micronucleus assay, and a *Hprt* gene mutation assay in CHO-K1cells; all the tests were negative. The available studies were all reliable and robust (conducted to GLP and OECD guidelines) and have not previously been evaluated by HSE.

The following key conclusion has been made with regard to the genotoxicity of metabolite 2,4-dichlorobenzoic acid: the metabolite is not genotoxic *in vitro*.

B.6.8.1.2. *metabolites of bixlozone selected for potential inclusion in the residue definitions – toxicological assessment*

The following metabolites of bixlozone were selected for potential inclusion in the residue definitions based on their significant occurrence in the plant and livestock metabolism studies (DAR Volume 3 Section B.7):

Compound name	Code (metabolism studies)	FMC Code[1]	Structure
5'-hydroxy-bixlozone	M289/3	FMC-077038	
5-hydroxy-bixlozone	M289/1	FMC-510226	
5-hydroxy-bixlozone-glucuronide	M465/1	N/A	
bixlozone-3-OH-propanamide	M275/1	FMC-510232	
bixlozone-3-OH-propanamide-sulfate	M355/1	N/A	
2,2-dimethyl-3-hydroxy propionic acid	M118/1	FMC-057089	
2,4-dichlorobenzoic acid [2]	M190/1	FMC-510224	
bixlozone-dimethyl-malonamide	M289/2	FMC-510233	
Dimethyl-malonic acid	M 132/1	FMC-043942	

[1] FMC Codes not generated for phase II metabolites

[2] Observed as the glycine conjugate: 2,4-dichlorohippuric acid

No specific toxicity studies are available on these metabolites; thus, in order to assess the toxicological properties of these metabolites all the available data (including data relating to bixlozone) were taken into account by HSE. These included:

- The presence of these metabolites in rat ADME and other relevant toxicity studies performed with bixlozone
- Structural similarity to bixlozone
- *In silico* genotoxicity assessment
- *In silico* assessment of general toxicity

- Publicly available data available on the metabolites

Presence of the selected metabolites in rat ADME and toxicity studies (*Table B 6.8.1.1*)

It is well accepted that the toxicity profile of plant/livestock metabolites which are major rat metabolites is 'covered' by the toxicity profile of the parent (i.e. active substance). A major rat metabolite is a metabolite contributing to 10 % or more of the administered dose (AD) in terms of total radioactive material recovered in urine in both sexes as detected in ADME studies (Guidance on the establishment of the residue definition for dietary risk assessment adopted 22 July 2016 - EFSA Journal 2016;14(12):4549).

The review of the ADME studies conducted with bixlozone shows that none of the above metabolites has been detected *in vivo* in rat urine at levels > 10% of the AD (Section **Error! Reference source not found.**). However, as shown in Table B 6.8.1.1 below, 2,4-dichlorobenzoic acid and 5-hydroxy-bixlozone can be regarded as major rat metabolites of bixlozone as they are direct precursors of 2,4-dichlorohippuric acid and 5-keto-hydrate-bixlozone respectively (see Figure B.6.1.1.1 in ADME section), which are present in rat urine at levels > 10% of the AD in both sexes (see Table B.6.1.1.5.3 in the ADME section).

2,4-dichlorobenzoic acid (CAS 50-84-0) is a putative *in vivo* rat metabolite considered to be covered via its downstream glycine conjugate 2,4-dichlorohippuric acid, the latter being recovered in urine at levels > 10 % of the AD in both sexes following single low dose oral exposure ([redacted], 2018b). On this basis, its toxicity profile could be considered 'covered' by the parent. However, specific data are available on this metabolite (see below). These data take precedence on the prediction considered above (see section below for a conclusion).

5-hydroxy-bixlozone is formed by simple hydroxylation of bixlozone and is the exclusive upstream metabolite of the major metabolite 5-keto-hydrate-bixlozone, the latter being formed by oxidation of 5-hydroxy-bixlozone. The metabolite 5-keto-hydrate-bixlozone has been consistently recovered in urine at levels of 14 - 35 % of the AD in both sexes following single and repeated oral exposure ([redacted], 2018b). Thus 5-hydroxy-bixlozone can be regarded as a major rat metabolite as it is the direct precursor of a major rat metabolite, 5-keto-hydrate-bixlozone.

In addition, the metabolite 5-hydroxy-bixlozone-glucuronide identified in the plant and livestock metabolism studies is an enzyme hydrolysable conjugate of 5-hydroxy-bixlozone arising from a detoxification pathway (glucuronidation) that would be expected to result in the rapid excretion of the molecule. Thus, 5-hydroxy-bixlozone-glucuronide is expected to be covered by its aglycon metabolite 5-hydroxy-bixlozone, which has been regarded as a major rat metabolite.

Overall, the toxicological properties of **5-hydroxy-bixlozone** and **5-hydroxy-bixlozone-glucuronide**, as major rat metabolites, can be considered to have been intrinsically tested in the toxicological studies undertaken with bixlozone and thus these metabolites can be considered of **equivalent toxicity to the parent substance** and potential candidates for inclusion in the Residue Definition from a toxicological perspective. If a risk assessment were to be required for 5-hydroxy-bixlozone and 5-hydroxy-bixlozone-glucuronide, the **dietary reference values of bixlozone** could be used.

Table B 6.8.1.1: Assessment of coverage of plant/livestock residue metabolites by the toxicological data on the parent bixlozone

Compound name	Code	FMC Code[1]	Occurrence in rat urine (both sexes) % of the AD	Covered by downstream major metabolite in rat urine?	Covered by toxicological studies on parent?
Bixlozone (F9600; parent)	-	FMC-057049	< 1 %	-	-
5'-hydroxy-bixlozone	M289/3	FMC-077038	Not detected	No	No
5-hydroxy-bixlozone	**M289/1**	**FMC-510226**	**0.4 - 1 %**	**Yes Covered by oxidised metabolite 5-keto-hydrate-bixlozone (14 - 35 % of the AD in both sexes)**	**Yes**
5-hydroxy-bixlozone-glucuronide	**M465/1**	**N/A**	**1 - 6%**	**Yes Covered by aglycon metabolite 5-hydroxy-bixlozone (major rat metabolite as direct precursor of major rat metabolite, 5-keto-hydrate-bixlozone)**	**Yes**
bixlozone-3-OH-propanamide	M275/1	FMC-510232	< 1 %	No	No
bixlozone-3-OH-propanamide-sulfate	M355/1	N/A	Not detected	No	No
2,2-dimethyl-3-hydroxy propionic acid	M118/1	FMC-057089	Not detected	No	No
2,4-dichlorobenzoic acid[2]	**M190/1**	**FMC-510224**	Not detected	**Yes Covered by downstream glycine conjugate 2,4-dichlorohippuric acid (11.7% and 14.5% of AD in males and females respectively)**	**Yes[3]**
Bixlozone-dimethyl-malonamide	M289/2	FMC-510233	2.4 - 6.7%	No	No
Dimethyl-malonic acid	M 132/1	FMC-043942	Not detected	No	No

[1] FMC Codes not generated for phase II metabolites

[2] Observed as the glycine conjugate: 2,4-dichlorohippuric acid

[3] In principle it is covered by parent, but as specific data showing it is more toxic than parent are available, the specific data take precedence

Read-across: Structural similarity of the selected metabolites with the parent (*Table B 6.8.1.2*)

With regard to evaluation of chemical similarity of selected metabolites to bixlozone, the general principles given by the EFSA Scientific Opinion on Evaluation of the Toxicological Relevance of Pesticide Metabolites for Dietary Risk Assessment (EFSA Journal 2012;10(07):2799) were followed, taking into consideration:

1) The metabolic steps that most likely do not lead to additional toxicity of the metabolites:

- Simple demethylation of the ring or side chain
- Simple hydroxylation of the ring system without any cleavage of the ring
- Hydroxylation of another ring position
- Conjugation of a metabolite with amino acid

2) Conjugated metabolites (O-glucuronides and sugar conjugates) being of similar or lower toxicity compared to their unconjugated products (due to cleavage in the human gastrointestinal tract).

The structural similarity was also assessed by analysing the metabolite structures with the general mechanistic profilers of the OECD QSAR TOOLBOX (version 4.4), which showed that the metabolites 5-hydroxy-bixlozone, 5'-hydroxy-bixlozone and bixlozone-3-OH-propanamide are the most similar compounds to bixlozone.

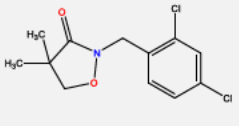
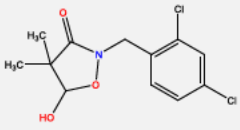
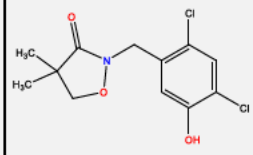
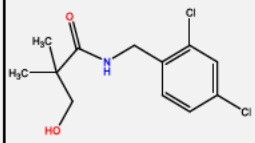
In general, slight structural and physical-chemical properties variations triggered by simple hydroxylation of the parent compound are not expected to generate significant changes in the toxicity profile of metabolites when compared with the parent. The metabolites 5-hydroxy-bixlozone and 5'-hydroxy-bixlozone only differ from bixlozone by one hydroxylation. The dice structural similarity[10] to bixlozone was calculated at 96.9% for 5 OH-bixlozone and 86.6% for 5'-hydroxy-bixlozone respectively and it is generally accepted that two structures are considered similar if the dice structural similarity is > 85%. Additionally, both metabolites have similar physical-chemical properties (log Kow and molecular weight) to bixlozone, as seen in Table B 6.8.1.2 below.

Comparative *in silico* analysis of 5-hydroxy-bixlozone did not flag any relevant additional alert compared to bixlozone (Wijeyesakere S.J. et al., 2020 Report number FMC-55114 & Sharon A. Jackson S.A., 2019 Report number 2018WHP-ISX4394). However, the hydroxylation of the phenyl ring of bixlozone to form 5'-hydroxy-bixlozone generates an additional phenol functional group not present in bixlozone, which triggers additional alerts for *in vitro* mutagenicity (Ames test) and skin sensitisation compared to bixlozone. Therefore, read-across to bixlozone is not considered appropriate for 5'-hydroxy-bixlozone at this stage and further assessment of the relevance of these alerts is warranted (please refer to the sub-sections below for further details).

The general mechanistic profilers of the OECD QSAR TOOLBOX also show that bixlozone-3-OH-propanamide is structurally similar to bixlozone with a dice structural similarity > 85% and comparable Log Kow and molecular weight. However, it is formed after hydroxylation and cleavage of the isoxazolidinone ring of bixlozone, which forms additional alcohol and carboxylic acid amide groups to those present in bixlozone. Moreover, this metabolite flagged an additional alert for skin sensitisation using the OECD QSAR Toolbox which is assessed for its toxicological relevance in the sections below. Thus, it cannot be ruled out that bixlozone-3-OH-propanamide has a different toxicity profile to bixlozone and so read-across to bixlozone is not considered appropriate at this stage.

[10] Dice structural similarity (%) was calculated via the fingerprint method using PubChem features using the OECD QSAR ToolBox (ver. 4.4). The dice coefficient is the number of features in common to both molecules relative to the average size of the total number of features present (range 0 to 1).

Table B 6.8.1.2: General mechanistic profilers from the OECD QSAR ToolBox (ver. 4.4) for bixlozone, 5-hydroxy-bixlozone, 5'-hydroxy-bixlozone and bixlozone-3-OH-propanamide.

Structure				
Chemical name	Bixlozone	5-hydroxy-bixlozone	5'-hydroxy-bixlozone	Bixlozone-3-OH-propanamide
SMILES	CC1(C)CON(Cc2ccc(Cl)cc2Cl)C1=O	CC1(C)C(O)ON(Cc2ccc(Cl)cc2Cl)C1=O	CC1(C)CON(Cc2cc(O)c(Cl)cc2Cl)C1=O	CC(C)(CO)C(=O)NCc1ccc(Cl)cc1Cl
log Kow	3.51	1.97	3.03	2.2
Molecular weight Da	274	290	290	276
Dice structural similarity to bixlozone (%)	100	96.9	86.6	NA
Organic functiona groups	Aromatic compound Aryl chloride Aryl halide Carboxylic acid derivative Halogen derivative Heterocyclic compound	Aromatic compound Aryl chloride Aryl halide Carboxylic acid derivative Halogen derivative Heterocyclic compound **Hydroxy compound** **Alcohol**	Aromatic compound Aryl chloride Aryl halide Carboxylic acid derivative Halogen derivative Heterocyclic compound **Hydroxy compound** **Phenol**	Aromatic compound Aryl chloride Aryl halide Carboxylic acid derivative Halogen derivative Heterocyclic compound **Hydroxy compound** **Alcohol** **Carboxylic acid amide**

ND: Not detected

***In silico* genotoxicity assessment of selected metabolites (**Table B 6.8.1.3**,** Table B 6.8.1.4 **&** Table B 6.8.1.5**)**

The genotoxicity potential of bixlozone was assessed according to the data requirements of Regulation 283/2013. Bixlozone did not induce gene mutations in bacteria or in mouse lymphoma cells *in vitro,* however it was clastogenic in Chinese hamster ovary (CHO-K1) cells *in vitro* when tested with metabolic activation (S9) (Section B.6.4.1.2). The clastogenic activity seen *in vitro* was not evident *in vivo* when tested up to the limit dose of 2000 mg/kg bw in a valid rat bone marrow micronucleus study. Overall, it was concluded that bixlozone is not genotoxic *in vivo*.

To predict the genotoxic potential of the metabolites of bixlozone, structural profiling was undertaken using the OECD QSAR ToolBox (version 4.4.1), along with two other predictive models: Derek Nexus (Lhasa Ltd), a knowledge-based system; and Sarah Nexus (Lhasa Ltd), a statistical tool. The applicant provided the predictions for gene mutation and chromosomal aberrations flagged by the metabolites and compared these to those of bixlozone (Wijeyesakere S.J. *et al*., 2020 Report number FMC-55114 & Sharon A. Jackson S.A., 2019 Report number 2018WHP-ISX4394). In addition, the toxic hazard of the metabolites was estimated by applying a decision tree approach using Cramer rules with Toxtree version 3.1.0. This data was reviewed by HSE.

(Q)SAR models used

The specifications of the (Q)SAR models used are presented below; the model validation reports in the (Q)SAR model report format (QMRF) for the knowledge based and statistical (QS)AR tools used in this assessment were downloaded from the EU Joint Research Centre QMRF database (https://qsardb.jrc.ec.europa.eu/qmrf/ - accessed June 2020) and copies of the QMRF reports were provided to HSE.

Data model	Data model source	Date predictions performed	Information on the QSAR model
OECD Toolbox v. 4.4.1	OECD & ECHA; developed by The Laboratory of Mathematical Chemistry (LMC)	August 2020	Structural profilers
Toxtree v. 3.1.0	Ideaconsult Ltd.	August 2020	Decision tree spproach
Derek Nexus v. 6.0.1	Lhasa Ltd.	August 2020	Knowledge-based
Sarah Nexus v. 3.0.0	Lhasa Ltd.	August 2020	Statistical-based

QSAR Toolbox (OECD) v. 4.4.1

The OECD QSAR Toolbox is a freely available software application intended to be used to fill gaps in toxicity and ecotoxicity data needed for assessing the hazards of chemicals. The Toolbox incorporates databases on chemical data (e.g. phys-chem properties), experimental toxicological and ecotoxicological data and estimated values from a large range of QSAR tools, together with incorporated QSAR modelling and Expert Systems, built within a regulatory application shell. The package therefore allows the user to perform a number of functions.

- Identify analogues for a chemical, retrieve experimental results available for those analogues and fill data gaps by read-across or trend analysis;
- Categorise large inventories of chemicals according to mechanisms or modes of action;
- Fill data gaps for any chemical by using the library of QSAR models;
- Evaluate the robustness of a potential analogue for read-across;
- Evaluate the appropriateness of a (Q)SAR model for filling a data gap for a particular target chemical;
- Build QSAR models.

For the purposes of this work, the toolbox was used to provide mechanistic and human health hazard profiling for a range of parameters as outlined in the table below.

Profile	Description
DNA binding by OASIS v 1.3	The profiler is based on AMES Mutagenicity model part of OASIS TIMES system. The scope of the profiler is to investigate presence of alerts within target molecules responsible for interaction with DNA. The profiling result outcome assigns a target to the corresponding structural alert, mechanistic alerts and domain
DNA binding by OECD	The profiler was created following the mapping of existing structural alerts for mutagenicity and carcinogenicity. The alerts cross six broad organic chemistry mechanisms and represent the most comprehensive listing of structural alerts for DNA binding by OECD currently available.
Toxic hazard classification by Cramer (with extensions)	Toxic hazard classification by Cramer profiler is built on the original paper of G.M. Cramer and R.A. Ford Estimation of toxic hazard - a decision tree approach. Food and Cosmetics Toxicology, Volume 16, Issue 6, December 1978, Page 255-276 Decision tree comprises 33 structural rules and place compounds in one of the three classes: low (Class I), intermediate(Class II) and high (Class III)
DNA alerts for AMES, MN and CA by OASIS v 1.3	The profiler is based on AMES Mutagenicity model part of OASIS TIMES system. The scope of this profiler is to investigate the presence of alerts within the target molecules responsible for interaction with DNA, especially related to Ames mutagenicity, Chromosomal aberration and Micronucleus tests. The profiling result outcome assigns a target to the corresponding structural alert, mechanistic alerts and domain.
In-vitro mutagenicity (Ames test) alerts by ISS	This profiler is based on the Mutagenicity/Carcinogenicity module of the software Toxtree. It works as a decision tree for estimating in vitro (Ames test) mutagenicity, based on a list of 30 structural alerts (SAs).

Profile	Description
In-vivo mutagenicity (micronucleus) alerts by ISS	This profiler is based on the ToxMic rule base of the software Toxtree. The SAs are molecular functional groups or substructures that are known to be linked to the induction of effects in the in vivo micronucleus assay.
Protein binding alerts for Chromosomal aberration by OASIS v1.1	The scope of this profiler is to investigate the ability of target molecules to elicit clastogenicity and aneugenicity. Functionalities which bring about steric (or electronic) hindrance in molecules and thus impede interactions with proteins are explicitly defined and associated with some of the alerts as "inhibition" masks.

Toxtree v. 3.1.0

Toxtree is a freely available software that uses a decision-tree approach to estimate the potential toxic hazard of a substance, based upon its molecular structure, and by applying a series of established rules. The structural alerts are available for inspection within the software.

Derek Nexus v. 6.0.1

Derek Nexus is a knowledge-based expert system designed to apply structure-activity relationships to compounds for which little or no data exists and hence to aid in the assessment of their potential toxicity. Derek Nexus was developed from Derek for Windows and is being continually refined as new knowledge becomes available. Any predictions made by Derek Nexus should be used in conjunction with other assessment techniques. As with all *in silico* modelling programs, the results of the predictions are reliant upon information that is available in databases, which occasionally may incorrectly predict a compound's toxicity.

The Derek Nexus knowledge base contains a large number of rules that associate a chemical structure with one or more toxicity endpoints. When a structural alert is identified a reasoning program assigns a probability to the expression of toxicity by the test compound. All the available end-points for mutagenicity and chromosomal damage in Derek were selected for profiling.

The applicant argued that while a validated, fit-for-purpose QSAR model for aneugenicity is currently not available, it was noteworthy that the data used to train and develop the knowledgebase for the chromosomal damage model in Derek Nexus (version 6.0.1 – Lhasa Ltd) included *in vitro* and *in vivo* micronucleus data (2448 compounds with *in vivo* micronucleus assay data and an additional 38 compounds with *in vitro* results). Thus, it is proposed that a positive flag in the "chromosomal damage" model in Derek Nexus can be used in a weight of evidence, to evaluate the numerical and structural chromosome damage potential of the metabolites of interest. HSE clarifies that (Q)SAR predictions for all genotoxicity endpoints are generally accepted for regulatory purposes when combined in a weight-of-evidence approach with any other useful information from grouping and read-across approaches and the presence of reactive toxicophore (which was addressed here by the structural profiling within the OECD QSAR Toolbox).

The levels of probability together with their definitions are given below:

Probability	Definition
Certain	There is proof that the proposition is true
Probable	There is at least one strong argument that the proposition is true and there are no arguments against it
Plausible	The weight of evidence supports the proposition
Equivocal	There is an equal weight of evidence for and against the proposition
Doubted	The weight of evidence opposes the proposition
Improbable	There is at least one argument that the proposition is false and there are no arguments that it is true
Impossible	There is proof that the proposition is false.
Open	There is no evidence that supports or opposes the proposition.
Contradicted	There is proof that the proposition is true and that the proposition is false.

Sarah Nexus v. 3.0.0

Sarah Nexus is a statistical software tool that provides mutagenicity predictions and it uses a unique, hierarchical, machine-learning methodology to build a model for Ames mutagenicity. No other endpoints are assessed using this software. Query structures which are imported into Sarah are standardised and then fragmented. These fragments are reviewed for activity versus inactivity based on (in)activity of training set examples. Sarah further refines the fragments by considering the similarity of the query structure to a training set of compounds. The structure standardisation in Nexus 2.2 uses a set of transform rules including, but not limited to, aromaticity perception, transforming tautomer and resonance forms, and removing salts. The aim of the standardisation is to interpret structures more accurately, in order to optimise predictions and minimise false signal strengths. The fragments are arranged into a network of hypotheses (or nodes) and the fragments which are perceived to be of a greater value contribute to an overall prediction of toxicity. Fragments may be of various sizes and can even overlap, ensuring greater accuracy in predictions.

The overall prediction is comprised of the following items:

- A conclusion about the Ames mutagenicity of a structure, and a confidence rating in the prediction.
- The fragments on which the prediction is based and relevant examples from the training set, ordered by structural similarity to the query.
- Additional compounds which are similar to the query but were not used in the prediction.
- Strain information for prediction results and additional information compounds.
- Contribution information for training set example compounds and additional compounds. This includes references, if available.

This high level of transparent information facilitates the expert review process.

Prediction	Definition
Positive	The query structure is predicted to be positive in a bacterial reverse mutation assay (Ames test).
Negative	The query structure is predicted to be negative in a bacterial reverse mutation assay (Ames test).
Equivocal	A strong argument cannot be made based on the training set compounds and any hypotheses generated for the query compound for either activity or inactivity in a bacterial reverse mutation assay (Ames test). In the absence of a strong overall signal, an equivocal call has been made.
Out of Domain	At least one atom present in a fragment of the query compound is not represented by any of the fragments in the training set used to build the model. As a result, the compound is outside the training dataset domain and an overall prediction is not possible.
Confidence The overall confidence for a prediction is determined from the confidence of the individual hypotheses activated by the structure. These are, in turn, based on the signal and the Tanimoto similarity of the nearest neighbours to the query structure used to build the hypothesis. In the absence of any hypotheses being activated by the query compound, the signal and Tanimoto similarity of the nearest neighbours from the entire training set are used to generate the overall confidence.	

Results of the structural profiling for genotoxicity using the OECD QSAR ToolBox

No alerts were flagged for bixlozone or any assessed metabolite (except 5'-hydroxy-bixlozone – see below) by the following profilers:

1. DNA binding by OASIS
2. DNA binding by OECD
3. Protein Binding by OASIS
4. Protein binding by OECD
5. DNA alerts for AMES, CA and MNT by OASIS
6. *In vitro* mutagenicity (Ames test) alerts by ISS

The metabolite 5'-hydroxy-bixlozone flagged an additional "protein binding alerts for in vitro chromosomal aberration by OASIS" (alert AN2 >> Michael addition to the quinoid type structures) compared to bixlozone owing to the possible metabolic activation of its phenol sub-structure (not present in bixlozone) to yield a facile-reactive quinone. The metabolite on the other hand did not flag any corresponding DNA binding alert or alerts for *in vivo* mutagenicity.

The alerts eliciting the chromosomal aberration (CA) models by OASIS account for interactions with DNA and/or proteins. Direct binding of chemicals to DNA is one of the underlying mechanisms that are responsible for CA mutagenicity. Disturbance of protein synthesis due to inhibition of topoisomerases and interaction of chemicals with nuclear proteins associated with DNA (e.g., histone proteins) are identified as additional mechanisms also leading to positive CA effect. Thus the protein binding alert is included as a second reactivity component (complementing DNA reactivity) in the *in vitro* Chromosomal aberrations OASIS TIMES mutagenicity model. Both DNA and protein binding profilers do not account for the inability of these alerts to be expressed due to electronic and steric hindrance.

The applicant provided an assessment of the relevance of the AN2 alert in 5'-hydroxy-bixlozone. Several compounds of the training set curated for this alert have a similar substituted phenol structure to 5'-hydroxy-bixlozone, such as the simple chemicals 4-chlorophenol (CAS: 106-48-9) and 2,4-dichlorophenol (CAS: 120-83-2). Both compounds were tested positive in an *in vitro* mammalian chromosome aberration test without metabolic activation (reference for 4-chlorophenol from the National Institute of Health Sciences – Japan[11] and for 2,4-dichlorophenol from Hilliard, CA et al. (1998) [12]). However, publicly available data show that 4-chlorophenol is neither clastogenic nor aneugenic in a modern *in vitro* micronucleus assay (available in the ECHA-REACH Registration dossier; https://echa.europa.eu/registration-dossier/-/registered-dossier/30700/7/7/2).

Moreover, the OECD Toolbox literature tab linked to the alert highlights that the presence of halogens in positions 3, 4 and 5 against a hydroxyl group will impede the access of the phenoxyl radical to the carbon-centered radical, limiting the formation of the intermediate dimer and the corresponding quinone. The applicant underlined that this is the case here for 5'-hydroxy-bixlozone since it has a chloro group para to the phenol along with additional steric hindrance from the isoxazolidinone fragment, suggesting that this mechanism is less likely to occur with this metabolite.

The applicant also highlighted that since 5'-hydroxy-bixlozone did not flag any corresponding DNA binding alert or alerts for *in vivo* mutagenicity, the weight of evidence is suggesting that the protein binding alert for in vitro CA in isolation is of low concern.

HSE further noted that the alert is not supported by Derek Nexus which retrieved no alert for 5'-hydroxy-bixlozone (see further below).

In conclusion, the weight of evidence indicates that this alert is of low toxicological relevance to the genotoxic potential of 5'-hydroxy-bixlozone and that overall this metabolite is unlikely to be genotoxic.

No other additional alerts were identified for the other metabolites compared to bixlozone.

Overall, no relevant additional alerts have been highlighted for any of the assessed metabolites compared to bixlozone using the OECD QSAR ToolBox.

[11] https://www.cdc.gov/niosh-rtecs/SK2AB980.htm

[12] Hilliard,CA et al ; ENVIRON. MOL. MUTAGEN. 31(4):316-326, 1998

Table B 6.8.1.3: Results of the structural profiling for genotoxicity using the OECD QSAR ToolBox and Toxtree

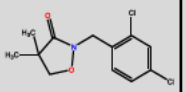
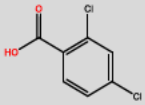
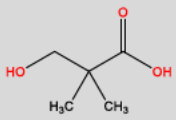
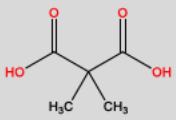
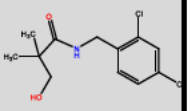
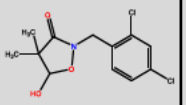
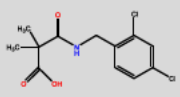
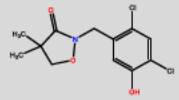
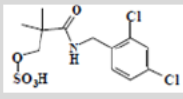
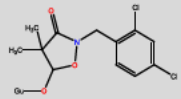
Structure					
CAS number	No CAS number	50-84-0	4835-90-9	595-46-0	No CAS number
Chemical name	Bixlozone (F9600)	2,4-Dichlorobenzoic acid	2,2-dimethyl-3-hydroxy propionic acid	Dimethyl-malonic acid	bixlozone 3-OH propanamide
Code	-	M190/1	M118/1	M 132/1	M275/1
SMILES	CC1(C)CON(Cc2ccc(Cl)cc2Cl)C1=O	OC(=O)c1ccc(Cl)cc1Cl	CC(C)(CO)C(O)=O	CC(C)(C(=O)O)C(=O)O	CC(C)(CO)C(=O)NCc1ccc(Cl)cc1Cl
OECD QSAR Toolbox Profilers					
Protein binding by OASIS	No alert found	No alert found	No alert found	No alert found	No alert found
DNA binding by OECD	No alert found	No alert found	No alert found	No alert found	No alert found
Toxic hazard classification by Cramer (extended)	High (Class III)	High (Class III)	High (Class III)	High (Class III)	High (Class III)
DNA binding by OASIS	No alert found	No alert found	No alert found	No alert found	No alert found
Protein binding by OECD	No alert found	No alert found	No alert found	No alert found	No alert found
in vivo mutagenicity (Micronucleus) alerts by ISS	H-acceptor-path3-H-acceptor	No alert found	H-acceptor-path3-H-acceptor	H-acceptor-path3-H-acceptor	H-acceptor-path3-H-acceptor
Protein binding alerts for Chromosomal aberration by OASIS	**No alert found**	No alert found	No alert found	No alert found	No alert found
in vitro mutagenicity (Ames test) alerts by ISS	No alert found	No alert found	No alert found	No alert found	No alert found
DNA alerts for AMES, CA and MNT by OASIS	No alert found	No alert found	No alert found	No alert found	No alert found
Toxtree – Toxic hazard by Cramer rules					
Toxic hazard classification by Cramer	High (Class III)	High (Class III)	Low (Class I)	Low (Class I)	High (Class III)

Table B 6.8.1.4: Results of the structural profiling for genotoxicity using the OECD QSAR ToolBox (continued)

Structure					
CAS number	No CAS number	No CAS number	No CAS number	No CAS number	No CAS number
Chemical name	5-OH bixlozone	bixlozone dimethyl malonamide	5'-OH bixlozone	bixlozone-3-OH-propanamide-sulfate	5-hydroxy-bixlozone_Glucuronide
Code	M289/1	M289/2	M289/3	M355/1	M465/1
SMILES	CC1(C)C(O)ON(Cc2ccc(Cl)cc2Cl)C1=O	CC(C)(C(=O)O)C(=O)NCc1ccc(Cl)cc1Cl	CC1(C)CON(Cc2cc(O)c(Cl)cc2Cl)C1=O	CC(C)(COS(O)(=O)=O)C(=O)NCc1ccc(Cl)cc1Cl	CC1(C)C(OC2OC(C(O)C(O)C2O)C(O)=O)ON(Cc2ccc(Cl)cc2Cl)C1=O
OECD QSAR Toolbox Profilers					
Protein binding by OASIS	No alert found	No alert found	No alert found	No alert found	No alert found
DNA binding by OECD	No alert found	No alert found	No alert found	No alert found	No alert found
Toxic hazard classification by Cramer (extended)	High (Class III)	High (Class III)	High (Class III)	High (Class III)	High (Class III)
DNA binding by OASIS	No alert found	No alert found	No alert found	No alert found	No alert found
Protein binding by OECD	No alert found	No alert found	No alert found	No alert found	No alert found
in vivo mutagenicity (Micronucleus) alerts by ISS	H-acceptor-path3-H-acceptor	H-acceptor-path3-H-acceptor	H-acceptor-path3-H-acceptor	H-acceptor-path3-H-acceptor	H-acceptor-path3-H-acceptor
Protein binding alerts for Chromosomal aberration by OASIS	No alert found	No alert found	**AN2** **AN2 >> Michael addition to the quinoid type structures** **AN2 >> Michael addition to the quinoid**	No alert found	No alert found
in vitro mutagenicity (Ames test) alerts by ISS	No alert found	No alert found	No alert found	No alert found	No alert found
DNA alerts for AMES, CA and MNT by OASIS	No alert found	No alert found	No alert found	No alert found	No alert found
Toxtree – Toxic hazard by Cramer rules					
Toxic hazard classification by Cramer	High (Class III)	High (Class III)	High (Class III)	High (Class III)	High (Class III)

QSAR analysis using Nexus knowledge-based and statistical-based QSAR models

Bixlozone and the metabolites of interest were assessed via the knowledge-based QSAR model Derek Nexus and the statistical-based QSAR model Sarah Nexus: no alerts for genotoxicity (mutagenicity or chromosomal damage by Derek and bacterial mutagenicity by Sarah) were flagged for either the parent bixlozone or any of the assessed metabolites. No misclassified/uncharacterised structural features were flagged by Derek Nexus for either bixlozone or the metabolites of interest.

Overall no relevant additional alerts have been highlighted for any of the assessed metabolites compared to bixlozone using Derek and Sarah Nexus.

Table B 6.8.1.5: Results of the QSAR analysis for genotoxicity using Nexus knowledge-based and statistical-based QSAR models

Compound name	Code	Derek Nexus		Sarah Nexus
		Mutagenicity	Chromosomal damage	Mutagenicity
Bixlozone (F9600; Parent)	-	Inactive	No alert	Negative
5'-OH bixlozone	M289/3	Inactive	No alert	Negative
5-OH bixlozone	M289/1	Inactive	No alert	Negative
bixlozone-3-OH-propanamide	M275/1	Inactive	No alert	Negative
2,2-dimethyl-3-hydroxy propionic acid	M118/1	Inactive	No alert	Negative
2,4-dichlorobenzoic acid	M190/1	Inactive	No alert	Negative
bixlozone dimethyl malonamide	M289/2	Inactive	No alert	Negative
Dimethyl-malonic acid	M 132/1	Inactive	No alert	Negative
bixlozone-3-OH-propanamide-sulfate	M355/1	Inactive	No alert	Negative
5-hydroxy-bixlozone-Glucuronide	M465/1	Inactive	No alert	Negative

Genotoxicity: Overall conclusions

Based on the weight of evidence presented above, no relevant additional alerts for genotoxicity have been highlighted for any of the assessed metabolites compared to bixlozone; thus it is concluded that none of the assessed metabolites are likely to be genotoxic.

Other information available to characterise the toxicity profile of metabolites of interest

5'-hydroxy-bixlozone (no CAS number, Code M289/3)

No publicly available toxicological data has been found for this metabolite. It is structurally closely related to bixlozone; its phenol functional group (not present in bixlozone) triggered an additional alert for skin sensitisation (alert 264 - polyhalogenated benzene; equivocal) using Derek Nexus compared to bixlozone. The biological relevance of this alert is uncertain since both positive and negative outcomes have been reported for various substituted benzene derivatives with an equivocal outcome. In addition, a skin sensitisation potential is of no relevance to the dietary route of exposure. No other alerts (carcinogenicity, organ toxicity, reproductive toxicity, irritation, skin and respiratory sensitisation, neurotoxicity) were identified. It should be noted however, that the reliability of QSAR predictions for these more complex endpoints is low.

5-hydroxy-bixlozone (no CAS number, Code M289/1)

No publicly available toxicological data has been found for this metabolite. It is structurally closely related to bixlozone since it only differs from bixlozone by one hydroxylation. Comparative *in silico* analysis of 5-hydroxy-bixlozone did not flag any relevant additional alert compared to bixlozone regarding carcinogenicity, organ toxicity, reproductive toxicity, irritation, skin and respiratory sensitisation, neurotoxicity. It should be noted however, that the reliability of QSAR predictions for these more complex endpoints is low.

Bixlozone-3-OH-propanamide (no CAS number, Code M275/1) and bixlozone-3-OH-propanamide-sulfate (no CAS number, Code M355/1)

No publicly available toxicological data has been found for bixlozone-3-OH-propanamide. A comparative *in silico* analysis of bixlozone-3-OH-propanamide to bixlozone flagged an alert for protein binding by OASIS (sensitisation) via Schiff base formation ('1,2-dicarbonyls and 1,3-dicarbonyls') using the OECD QSAR Toolbox. The assessment of the relevance of this alert showed that bixlozone-3-OH-propanamide falls outside the scope of the alert as its R groups are not hydrogen or carbon, as defined by the alert description, but rather 'O' and 'N". Moreover, the alert was not supported by Derek Nexus for which the prediction was negative. No other alerts were identified.

Overall, based on the limited information available, bixlozone-3-OH-propanamide is not predicted to have a more severe toxicity profile compared to bixlozone for the toxicity hazards considered (carcinogenicity, organ toxicity, reproductive toxicity, irritation, skin and respiratory sensitisation, neurotoxicity). This conclusion is applicable to its conjugated form, bixlozone-3-OH-propanamide-sulfate. It should be noted however, that the reliability of QSAR predictions for these more complex endpoints is low.

2,2-dimethyl-3-hydroxy propionic acid (CAS 4835-90-9, Code M118/1)

There is no harmonised classification under Regulation (EC) N°1272/2008 (Annex VI to the CLP Regulation) or registration dossier available for this metabolite, however the notified classifications for Human Health effects indicate the following hazards:

Notified classification and labelling according to CLP criteria on the ECHA database[13]:

Classification	Hazard statement	GCL from regulation 1272/2008
Skin irritation 2	H315	10%
Eye damage 1	H318	≥ 1 % but < 3 % for eye irritation 2 ≥ 3 % for eye damage 1
STOT SE 3	H335 (respiratory irritation)	20%

Comparative *in silico* analysis indicated no increased hazard regarding genotoxicity (Ames test, chromosome aberration and micronucleus tests), carcinogenicity, skin sensitisation and skin / eye irritation when compared to the active substance bixlozone by all models used. It should be noted however, that the reliability of QSAR predictions for these more complex endpoints is low.

Overall, based on the limited information available, 2,2-dimethyl-3-hydroxy propionic acid has a more severe toxicity profile compared to bixlozone, however the local effects assigned to this metabolite are of no relevance to the dietary route of exposure.

2,4-dichlorobenzoic acid (CAS 50-84-0, Code M190/1)

Although this metabolite was identified as a major metabolite in the rat (see above), it is also a common chemical which structurally differs significantly from bixlozone; thus, its toxicity profile was further characterised using various sources of information such as publicly available data and comparative *in silico* analysis for genotoxicity (Ames test, chromosome aberration and micronucleus tests), carcinogenicity, skin sensitisation and skin / eye irritation. For more details on the *in silico* tools used and the toxicity hazards predicted please refer to Volume 4 of the DAR (2,4-DCA is also an impurity).

No harmonised classification under Regulation (EC) N°1272/2008 (Annex VI to the CLP Regulation) or registration dossier is available for this metabolite however the notified classifications for Human Health effects indicate the following hazards:

[13] https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/39307

Notified classification and labelling according to CLP criteria on the ECHA database[14]:

Classification	Hazard statement	Generic cut-off criteria and GCL from regulation 1272/2008
Acute tox 4	H302 (oral)	1 %
Skin irritation 2	H315	10 %
Eye irritation 2	H319	10 %
STOT SE 3	H335 (respiratory irritation)	20 %

Additional information has been found supporting the acute oral classification (based upon a mouse acute oral LD_{50} of 830 mg/kg bw) and regarding acute toxicity by the subcutaneous route on the ChemID Plus database[15]:

ChemID Plus toxicity information on 2,4-dichlorobenzoic acid

Organism	Test Type	Route	Reported Dose (Normalized Dose)	Effect	Source
mouse	Acute LD_{50}	oral	830mg/kg (830mg/kg)	Behavioural effects: • ataxia • somnolence (general depressed activity Lungs, thorax, or respiration: dyspnoea	Shokuhin Eiseigaku Zasshi. Food Hygiene Journal. Vol. 20, Pg. 332, 1979.
mouse	Acute LD_{50}	subcutaneous	1200mg/kg (1200mg/kg)	Behavioural effects: • muscle weakness • altered sleep time (including change in righting reflex) Lungs, thorax, or respiration: dyspnoea	Biochemical Pharmacology. Vol. 13, Pg. 1538, 1964.

The comparative (Q)SAR analysis did not indicate any relevant additional alerts for genotoxicity for 2,4-dichlorobenzoic acid compared to bixlozone. In addition, since 2,4-dichlorobenzoic acid is a groundwater metabolite, an *in vitro* battery of genotoxicity tests has been generated by the applicant and submitted to the UK.

The metabolite was tested in an *in vitro* Salmonella typhimurium reverse mutation (Ames) assay, an *in vitro* micronucleus assay, and a Hprt gene mutation assay in CHO-K1cells; all the tests were conducted to GLP and OECD guidelines, and were negative. Thus, the fluoxapiprolin metabolite 2,4-dichlorobenzoic acid is concluded not to be genotoxic *in vitro*.

The OECD QSAR Toolbox however predicted 2,4-dichlorobenzoic acid to be an irritant according to the skin irritation/corrosion inclusion/exclusion rules and the eye irritation/corrosion inclusion/exclusion rules by BfR; this result is consistent with the publicly available data indicating 2,4-dichlorobenzoic acid being a skin and eye irritant Category 2. Regarding other toxicity endpoints a repeated-dose HESS alert linked to hepatotoxicity was flagged using the OECD QSAR Toolbox. This alert is mainly used for screening purposes and chemical category building when no ranking is generated in the result. It is also noted that 2,4-dichlorobenzoic acid has low structural similarity to the representative compounds pirprofen (CAS 31793-07-4) and furosemide (CAS 54-31-9) included in the training set for this alert. Furthermore, the alert is not supported by other *in silico* tools with any hepatotoxicity alerts generated from the Derek Nexus model. Therefore, this alert is overall considered to be of low biological relevance. No other alerts were identified.

Overall, 2,4-dichlorobenzoic acid appears to have a more severe toxicity profile compared to bixlozone. Although the local effects assigned to this metabolite are of no relevance to the dietary route of exposure, the acute oral hazard (mouse LD_{50} of 830 mg/kg bw) cannot be disregarded. Even though 2,4-dichlorobenzoic acid, as a major rat metabolite, could be considered 'covered' by the parent (see above), specific data on the substance should take precedence on a prediction. Bixlozone has an acute oral LD_{50} of > 2000 mg/kg bw in the rat (Merrill, 2014a). Despite the uncertainties in the species differences and in the reliability of the mouse LD_{50}

[14] https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/88971

[15] ChemID Plus URL: https://chem.nlm.nih.gov/chemidplus/sid/0000050840

study, these data indicate that 2,4-dichlorobenzoic acid may be approximately 2-fold more toxic than bixlozone (by taking into account that it is also a major rat metabolite of bixlozone, potentially covered by parent).

On this basis, it is concluded that **2,4-dichlorobenzoic acid is more toxic than the parent** and a likely candidate for inclusion in the Residue Definition from a toxicological perspective. If a risk assessment were to be required, the **dietary reference values of bixlozone should be used, adjusted for a relative potency factor of 2**.

Bixlozone-dimethyl-malonamide (no CAS number, Code M289/2)

No publicly available toxicological data has been found for this metabolite. A comparative *in silico* analysis of bixlozone-dimethyl-malonamide to bixlozone flagged the same alert for protein binding by OASIS (sensitisation) via Schiff base formation ('1,2-dicarbonyls and 1,3-dicarbonyls') using the OECD QSAR Toolbox identified for bixlozone-3-OH-propanamide. It was noted that here again bixlozone-3-OH-malonamide falls outside the scope of the alert as its R groups are not hydrogen or carbon, as defined by the alert description, but rather 'O' and 'N". No other alerts were identified.

Overall, based on the limited information available, bixlozone-dimethyl-malonamide is not predicted to have a more severe toxicity profile compared to bixlozone and its upstream metabolite bixlozone-3-OH-propanamide for the toxicity hazards considered (carcinogenicity, organ toxicity, reproductive toxicity, irritation, skin and respiratory sensitisation, neurotoxicity). It should be noted however, that the reliability of QSAR predictions for these more complex endpoints is low.

Dimethyl-malonic acid (CAS 595-46-0, Code M 132/1)

No harmonised classification under Regulation (EC) N°1272/2008 (Annex VI to the CLP Regulation) or registration dossier is available for this metabolite, however the notified classifications for Human Health effects indicate the following hazards:

Notified classification and labelling according to CLP criteria on the ECHA database[16]:

Classification	Hazard statement	GCL from regulation 1272/2008
Skin irritation 2	H315	10%
Eye irritation 2	H319	10%
STOT SE 3	H335 (respiratory irritation)	20%

A comparative *in silico* analysis of dimethyl-malonic acid to bixlozone flagged an alert for protein binding by OASIS (sensitisation) via Schiff base formation ('1,2-dicarbonyls and 1,3-dicarbonyls') using the OECD QSAR Toolbox. An evaluation of the OECD toolbox documentation showed that dimethyl-malonic acid falls outside the scope of the alert as its R groups are not hydrogen or carbon, as defined by the alert description, but rather 'O' and 'N". Moreover, the alert was not supported by Derek Nexus for which the prediction was negative. No other alerts were identified (carcinogenicity, organ toxicity, reproductive toxicity, irritation, skin and respiratory sensitisation, neurotoxicity). It should be noted however, that the reliability of QSAR predictions for these more complex endpoints is low.

Overall, based on the limited information available, dimethyl-malonic acid appears to have a more severe toxicity profile compared to bixlozone, however the local effects assigned to this metabolite are of no relevance to the dietary route of exposure.

Overall conclusion on the toxicological assessment of selected metabolites (Table B 6.8.1.6**)**

The metabolite **5'-hydroxy-bixlozone** (no CAS number, Code M289/3) is not a major metabolite in rats. Although it is structurally very similar to bixlozone (it only differs from it by the presence of an additional hydroxy group on the phenyl ring) as confirmed by the comparative in silico analysis, it would be more prudent not to assume equivalence with the parent in relation to general toxicity as the reliability of QSAR predictions for complex endpoints is generally low. Having excluded genotoxicity by QSAR analysis, if a risk assessment were to be required for **5'-hydroxy-bixlozone, the Cramer class III TTC chronic value of 1.5 µg/kg bw/day and acute value of 5 µg/kg bw[17]** could be used in a conservative first-tier assessment.

The metabolite **5-hydroxy-bixlozone** (no CAS number, Code M289/1) is a putative major rat metabolite considered to be covered via its downstream metabolite 5-keto-hydrate-bixlozone. On this basis, its toxicity profile could be considered 'covered' by the parent. It is structurally very similar to bixlozone since it only differs from it by the presence of an additional hydroxy group on the isoxazolidinone ring. No additional *in silico* alerts were flagged for this metabolite for genotoxicity or general toxicity hazards compared to bixlozone. In addition, the conjugated form of 5-hydroxy-bixlozone (5-hydroxy-bixlozone-glucuronide) is expected to have a comparable or less severe toxicity profile than 5-hydroxy-bixlozone. Overall, the toxicological properties of **5-hydroxy-bixlozone** and **5-hydroxy-bixlozone-glucuronide**, as major rat metabolites, can be considered to have been intrinsically tested in the toxicological studies undertaken with bixlozone and thus these metabolites can be considered of **equivalent toxicity to the parent substance** and potential candidates for inclusion in the Residue Definition from a toxicological perspective. If a risk assessment were to be required for 5-hydroxy-bixlozone and 5-hydroxy-bixlozone-glucuronide, the **dietary reference values of bixlozone** could be used.

[16] https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/88971

[17] EFSA (2012) Scientific Opinion on Evaluation of the Toxicological Relevance of Pesticide Metabolites for Dietary Risk Assessment, EFSA Journal 2012;10(07):2799

The metabolite **bixlozone-3-OH-propanamide** (no CAS number, Code M275/1) shares some structural similarity to bixlozone; however additional alcohol and carboxylic acid amide functional groups are formed when the isoxazolidinone ring of bixlozone is opened up. Although no additional *in silico* alerts were flagged for this metabolite for genotoxicity or general toxicity compared to bixlozone, the reliability of QSAR predictions for complex general toxicity endpoints is low. Its conjugate form, bixlozone-3-OH-propanamide-sulfate (no CAS number, Code M355/1) is expected to have comparable or less severe toxicity. Its downstream metabolite bixlozone-dimethyl-malonamide (no CAS number, Code M289/2) is structurally close to bixlozone-3-OH-propanamide; both shared the same comparative *in silico* findings. None of these metabolites is a major rat metabolite. However, having excluded genotoxicity by QSAR analysis, if a risk assessment were to be required for **bixlozone-3-OH-propanamide, bixlozone-3-OH-propanamide-sulfate and bixlozone-dimethyl-malonamide, the Cramer class III TTC chronic value of 1.5 µg/kg bw/day and acute value of 5 µg/kg bw** could be used in a conservative first-tier assessment. Given their close structural similarity, a combined risk assessment of these three metabolites against the TTC values should be performed, if required.

The metabolite **2,4-dichlorobenzoic acid** (CAS 50-84-0, Code M190/1) is a putative major rat metabolite considered to be covered via its downstream glycine conjugate 2,4-dichlorohippuric acid, the latter being recovered in rat urine at levels > 10 % of the AD in both sexes following single low dose oral exposure. On this basis, its toxicity profile could be considered 'covered' by the parent. However, specific data are available on this metabolite. These data take precedence on the kinetic prediction and indicate that 2,4-dichlorobenzoic acid is not genotoxic in vitro in modern studies but may be approximately 2-fold more toxic than bixlozone. On this basis, it is concluded that **2,4-dichlorobenzoic acid (M190/1) is more toxic than the parent** and a likely candidate for inclusion in the Residue Definition from a toxicological perspective. If a risk assessment were to be required, **the dietary acute and chronic reference values of bixlozone should be used**, adjusting the residue estimate of 2,4-dichlorobenzoic acid for a relative potency factor of 2. In addition, a modifying factor of 1.435 should also be applied to account for the molecular weight conversion between the parent and the metabolite. This will allow to express 2,4-dichlorobenzoic acid into parent bixlozone equivalents.

The two metabolites **2,2-dimethyl-3-hydroxy propionic acid** (CAS 4835-90-9, Code **M118/1**) and **dimethyl-malonic acid** (CAS 595-46-0, Code **M 132/1**) are not structurally similar to bixlozone but are closely related to each other. Both substances are not major rat metabolites. No additional *in silico* alerts were flagged for these metabolites for genotoxicity compared to bixlozone; however, they both have classification notifications indicating a more severe toxicity profile compared to bixlozone. These general toxicity hazards (local irritant effects on skin, eye and respiratory tract) are nevertheless of no relevance to the dietary route of exposure. In the residues assessment in the DAR (Vol 1, section 2.7.4 and 2.7.3), it is noted how the applicant has proposed that residues of M118/1 have occurred in a number of control and treated crop samples from the field trials, and indicated that this was due to its natural occurrence. HSE requested additional information to justify the natural occurrence of the metabolites in food. The applicant responded that both metabolites M118/and M132/1 are small natural molecules that should be considered benign in nature and a review publication was submitted to support such a statement (Rezanka T, Kolouchova I, Cejkova A and Sigler K. Biosynthesis and metabolic pathways of pivalic acid. Appl. Micobiol. Biotechnol. 95, 1371-1376 (2012)). In the review the authors referred to literature that indicates that 2,2-dimethyl-3-hydroxy propionic acid (named pivalic acid in the review) can be biosynthesised by some bacteria to be incorporated into fatty acids (genera Alicyclobacillus, Rhodococcus, and Streptomyces) and into the antibiotic avermectin (Streptomyces avermitilis). They concluded that since pivalic acid is a starter unit in fatty acid biosynthesis, it must be presumed that pivalic acid is a natural compound. HSE considers that presence of pivalic acid in bacteria does not make this metabolite a naturally derived component of food and that the evidence provided does not rule out that 2,2-dimethyl-3-hydroxy propionic acid can have intrinsic toxicological properties that may affect human health.

Regarding Cramer Class predictions, HSE noted that both metabolites were assigned TTC Cramer Class I in the current online version of Toxtree (https://apps.ideaconsult.net/data/ui/toxtree). However, in the report provided by the applicant (Wijeyesakere S.J. et al., 2020 Report number FMC-55114) it was stated that both metabolites should be assigned TTC Cramer Class III according to the extended Cramer classification profiler implemented within the OECD QSAR Toolbox (version 4.4.1) and based on both compounds having a complex chemical structure and not being a normal component of food.

In order to address the inconsistencies described above, HSE requested the applicant to present any further data or justification to consider the Cramer Class assignation further. The applicant reviewed the rule interpretation of the Cramer decision tree from Toxtree (ver. 3.1.0) and the OECD QSAR Toolbox (ver. 4.4) and estimated that the discrepancies seen between the results from both tools could be due to differences in answering one of the Cramer decision tree questions. In the case of the metabolites 2,2-dimethyl-3-hydroxy propionic acid and dimethyl-malonic acid, the difference arises as a result of the interpretation of Cramer Rule # 20 (rule title:

aliphatic with some functional groups) which was answered with a "YES' by Toxtree but with a 'NO" by the OECD QSAR Toolbox. Since both metabolites are fitting with the rule explanation that they both are "simply branched aliphatic compounds containing four or less alcohol or acid functional groups" a "YES" response is concluded to be the most appropriate response. Overall, while both models are useful tools to implement the Cramer decision tree, following expert judgment for rule interpretation[18] the applicant concluded that the OECD QSAR Toolbox classification was incorrect and that Cramer Class I should be assigned to both metabolites 2,2-dimethyl-3-hydroxy propionic acid and dimethyl-malonic acid.

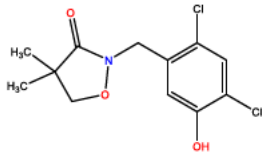
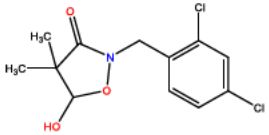
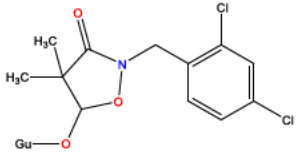
HSE reviewed the case provided by the applicant and considered it acceptable. Having excluded a genotoxicity potential, the limited toxicological data available for the metabolites show the presence of local effects of no relevance to the dietary route of exposure. The review of the toxic hazard of the metabolites estimated using Cramer rules indicated that the Cramer Class I is the most appropriate assignment to both metabolites 2,2-dimethyl-3-hydroxy propionic acid and dimethyl-malonic acid. Cramer class I substances are simple chemical structures for which efficient modes of metabolism exist, suggesting a low order of oral toxicity. There was insufficient evidence provided to ascertain they are naturally derived component of food and the evidence provided did not rule out they can have intrinsic toxicological properties that may affect human health. Nevertheless, the TTC chronic value for Cramer class I substances is at 30 µg/kg bw/day, one order of magnitude lower than the ADI of 0.3 mg/kg bw/day (300 µg/kg bw/day) set for bixlozone. Overall, following a weight of evidence approach, the TTC Cramer Class I chronic value is considered conservative and could be used in a first-tier assessment for these two metabolites.

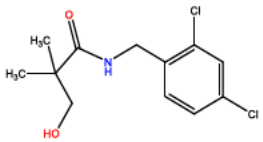
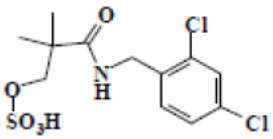
In conclusion, having excluded genotoxicity by QSAR analysis, if a risk assessment were to be required for **2-dimethyl-3-hydroxy propionic acid and dimethyl-malonic acid, the Cramer class I TTC chronic value of 30 µg/kg bw/day** could be used in a conservative first-tier assessment. This TTC value can also be used for the acute exposure assessment for these metabolites (when performing an initial 'screen' versus the TTC (CCI)). Given their close structural similarity, a combined risk assessment of these two metabolites against the TTC value should be performed, if required.

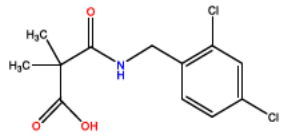
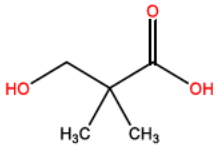
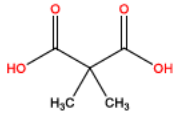
The table below summarises the available toxicological data for the metabolites selected for potential inclusion in the residue definitions.

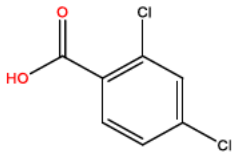
[18] Bhatia, S., et. al., (2015). Comparison of Cramer classification between Toxtree, the OECD QSAR Toolbox and expert judgment. *Regulatory Toxicology and Pharmacology*, *71*(1), 52–62.

Table B 6.8.1.6: Summary table on the assessment of the toxicological profile of metabolites

Name, metabolism code, FMC code and smiles	Structure	Percentage of the applied/absorbed dose in urine in both sexes in ADME studies	Genotoxicity conclusion and basis (endpoints). Experimental data/QSAR/grouping and Read-across/ covered by parent	General toxicity conclusion and basis (endpoints) Experimental data/QSAR/grouping and Read-across/covered by parent. Reference values (UF and basis)
5'-hydroxy-bixlozone **M289/3** **FMC-077038**		Not detected	Negative genotoxicity QSAR prediction.	Hydroxylated parent; similar to parent; added phenol functional group, but reliability of QSAR general toxicity predictions low. Toxicity of 5'-hydroxy-bixlozone not covered by toxicity data of parent bixlozone. **Cramer Class III TTC values**
5-hydroxy-bixlozone **M289/1** **FMC-510226**		0.4 - 1 % Major rat metabolite - covered by downstream major metabolite 5-keto-hydrate-bixlozone (14 - 35 % of the AD in both sexes)	Negative genotoxicity QSAR prediction.	Hydroxylated parent; similar to parent. Toxicity profile expected to be comparable to bixlozone and covered by toxicity data of parent bixlozone (as major rat metabolite). **Dietary reference values of bixlozone could be used if required.**
5-hydroxy-bixlozone-glucuronide **M465/1** **N/A**		1 – 6 %	Negative genotoxicity QSAR prediction.	Toxicity profile expected to be comparable to its aglycon -5-hydroxy-bixlozone. Toxicity of 5-hydroxy-bixlozone-glucuronide covered by 5-OH- bixlozone. **Dietary reference values of bixlozone could be used if required.**

Name, metabolism code, FMC code and smiles	Structure	Percentage of the applied/absorbed dose in urine in both sexes in ADME studies	Genotoxicity conclusion and basis (endpoints). Experimental data/QSAR/grouping and Read-across/ covered by parent	General toxicity conclusion and basis (endpoints) Experimental data/QSAR/grouping and Read-across/covered by parent. Reference values (UF and basis)
bixlozone-3-OH-propanamide **M275/1** **FMC-510232**		< 1 %	Negative genotoxicity QSAR prediction.	Structural similarity to parent but contains additional alcohol and carboxylic acid amide groups to those present in bixlozone. Difference in general toxicity profile compared with bixlozone cannot be ruled out as reliability of QSAR general toxicity predictions low. Toxicity of bixlozone-3-OH-propanamide is not covered by the toxicity data of the parent (as not a major rat metabolite). **Cramer Class III TTC values could be used if required.**
bixlozone-3-OH-propanamide-sulfate **M355/1** **N/A**		Not detected	Negative genotoxicity QSAR prediction.	Conjugated form of bixlozone-3-OH-propanamide. Toxicity of bixlozone-3-OH-propanamide-sulphate is covered by bixlozone-3-OH-propanamide Not a major rat metabolite **Cramer Class III TTC values could be used if required.**

Name, metabolism code, FMC code and smiles	Structure	Percentage of the applied/absorbed dose in urine in both sexes in ADME studies	Genotoxicity conclusion and basis (endpoints). Experimental data/QSAR/grouping and Read-across/ covered by parent	General toxicity conclusion and basis (endpoints) Experimental data/QSAR/grouping and Read-across/covered by parent. Reference values (UF and basis)
bixlozone-dimethyl-malonamide **M289/2** **FMC-510233**		2.4 -6.7%	Negative genotoxicity QSAR prediction.	Structural similarity to upstream metabolite bixlozone-3-OH-propanamide. Toxicity of bixlozone-dimethyl-malonamide is not covered by the toxicity data of the parent (as not major rat metabolite). **Cramer Class III TTC values could be used if required.**
2,2-dimethyl-3-hydroxy propionic acid **M118/1** **FMC-057089** **CAS 4835-90-9**		Not detected	Negative genotoxicity QSAR prediction.	Not structurally similar to parent. Assigned classification notifications indicate more severe local toxicity profile compared to bixlozone, but no relevance to dietary route of exposure Toxicity of dimethyl-malonic acid is not covered by the toxicity data of the parent (as not a major rat metabolite). **Cramer Class I TTC values could be used if required.**
Dimethyl-malonic acid **M 132/1** **FMC-043942**		Not detected	Negative genotoxicity QSAR prediction.	Not structurally similar to parent. Assigned classification notifications indicate more severe local toxicity profile compared to bixlozone, of no relevance to dietary route of exposure.

Name, metabolism code, FMC code and smiles	Structure	Percentage of the applied/absorbed dose in urine in both sexes in ADME studies	Genotoxicity conclusion and basis (endpoints). Experimental data/QSAR/grouping and Read-across/ covered by parent	General toxicity conclusion and basis (endpoints) Experimental data/QSAR/grouping and Read-across/covered by parent. Reference values (UF and basis)
CAS 595-46-0				Toxicity of dimethyl-malonic acid is not covered by the toxicity data of the parent (as not a major rat metabolite). **Cramer Class I TTC values could be used if required.**
2,4-dichlorobenzoic acid **M190/1** **FMC-510224** **CAS 50-84-0**		Major rat metabolite > 10 % (covered via its downstream glycine conjugate 2,4-dichlorohippuric acid, the latter being recovered in urine at levels > 10 % of the AD in both sexes following single oral exposure)	Experimental data (*in vitro*): Ames test, mammalian cell micronucleus test in human peripheral lymphocytes and HPRT mutation test using Chinese hamster ovary cells. All tests were negative. In addition, negative genotoxicity QSAR prediction.	Major rat metabolite; however, specific data available indicating more severe toxicity than parent (approximately 2-fold). **Dietary acute and chronic reference values of bixlozone should be used if required, adjusting the residue estimate of 2,4-dichlorobenzoic acid for a relative potency factor of 2. In addition, a modifying factor of 1.435 should also be applied to account for the molecular weight conversion between the parent and the metabolite. This will allow to express 2,4-dichlorobenzoic acid into parent bixlozone equivalents.**

B.6.8.2. Supplementary studies on the active substance

Palatability studies in mice, rats and dogs over 7-day (diet) repeated administration have been conducted and they are summarised in Section **Error! Reference source not found.** for repeated-dose toxicity. These studies concluded that there were transient palatability-related issues seen in the dog, but not in the rat and mouse.

No other supplementary studies on the active substance have been submitted.

B.6.8.2.1. *Immunotoxicity*

No specific immunotoxicity study conducted with bixlozone is available. Potential biomarkers of immunotoxicity have been measured in the existing toxicology studies including short-term, chronic, carcinogenic and reproductive toxicity studies conducted in multiple species. In all of the relevant toxicology studies conducted, immunotoxicology parameters including haematology (blood neutrophil, mononuclear cells, eosinophils, white blood cell (WBC) counts), clinical chemistry (albumin and globin ratio), organ weights (spleen, thymus), gross and histopathological examination of immunological organs (thymus, spleen, bone marrow and lymph nodes) have been included.

There was no indication that bixlozone has effects on the immune system in experimental animals from any of the studies conducted. Overall, HSE concludes that bixlozone does not affect the immune system, and a specific *in vivo* immunotoxicity study is not required.

B.6.8.3. Studies on endocrine disruption

This section includes an assessment of the adverse effects potentially related to an endocrine mode of action as observed in the regulatory toxicological studies conducted with bixlozone. Since bixlozone is a new substance, there are no *in vitro* endocrine activity assays reported under ToxCast and none have been conducted by the Applicant.

The definition of an endocrine disruptor (ED) is based on the WHO/IPCS (2002) definition and the assessment of endocrine disruption is based on the criteria for endocrine disruption (Commission Regulation (EU) 2018/605 of 19 April 2018). The criteria as listed in Annex II point 3.6.5 are as follows:

'From 20 October 2018, an active substance, safener or synergist shall be considered as having endocrine disrupting properties that may cause adverse effect in humans if, based on points (1) to (4) of the sixth paragraph, it is a substance that meets all of the following criteria, unless there is evidence demonstrating that the adverse effects identified are not relevant to humans:

1) *it shows an adverse effect in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;*
2) *it has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system;*
3) *the adverse effect is a consequence of the endocrine mode of action.'*

For the evaluation of the first criterion, it has to be determined whether adverse effects potentially related to an endocrine mode of action are observed. In this section the evaluation of effects on reproductive and endocrine related organs from all valid repeated-dose toxicity, long-term and reproductive toxicity studies are compiled and the adversity and specificity of the observed effects is assessed. For the evaluation of the second criterion (if applicable), the available *in vitro* endocrine activity assays are evaluated. To fulfil the third part of the criteria, an assessment as to whether the determined adverse effects are a consequence of an endocrine-mediated mechanism is performed.

The present assessment for the new active substance bixlozone follows the ECHA/EFSA/JRC guidance for the identification of endocrine disruptors in the context of Regulations (EU) 528/2012 and (EC) No 1107/2009 (EFSA Journal 2018;16(6):5311). The guidance proposes a workflow for assessing the endocrine disrupting

properties of pesticides and biocides, which starts by collecting all available data on bixlozone and assembling them into lines of evidence in the format of an Excel File (Appendix E of the guidance). This is followed by the analysis of the evidence which includes a decision tree with different possible scenarios to conclude whether a substance does not meet the ED criteria, additional information is needed, or a MOA analysis is required to conclude on the ED properties. The MOA analysis step aims to establish if there is a biologically plausible link between observed adverse effects and endocrine activity. Finally, the conclusion as to whether the ED criteria are met with respect to humans is drawn and transparently documented, including the remaining uncertainties.

B.6.8.3.1. *Evaluation of bixlozone toxicology– gather all information*

Based on Commission Regulation (EU) No 2018/605, the assessment of the potential for endocrine disruption of a substance should be based on a Weight of Evidence (WoE) approach and information can be obtained from existing data, read-across from structurally similar chemicals, *in silico* tools, *in vitro* and *in vivo* screening assays and/or from mechanistic studies. The assays appropriate for use in the WoE determination are discussed in the OECD Conceptual Framework (CF) for Testing and Assessment of Endocrine Disruptors (OECD Revised Guidance Document 150, 2018b). Using combinations of Level 1- Level 5 assays, endocrine disruptors can be identified according to their adverse effects on apical endpoints (Level 4 and 5 studies), taking into account severity, reversibility, potency and consistency, and endocrine activity (Level 2 and 3 studies). Currently, the most complete testing battery exists for oestrogen, androgen, thyroid and steroidogenesis (EATS) modalities, while non-EATS modalities will require further development in the future to allow reliable assessments.

The Level 1 to Level 5 toxicological information available for bixlozone regarding its potential for endocrine disruption is presented below:

1. OECD CF 150 Level 1 – Existing or new Non-test Information

ADME

Bixlozone is well absorbed, extensively distributed and metabolised and is excreted rapidly in rats after oral administration with the urine as the main route of excretion ([redacted], 2016). The oral bioavailability was found to be around 70-80 % with a slightly greater bioavailability seen in females compared to males. There was no evidence of bioaccumulation following repeated oral dosing (14 days) in the rat ([redacted], 2016). More details can be found in Section **Error! Reference source not found.** of the DAR.

OSAR

The OECD QSAR Toolbox (version 4. 3.1) was used to predict the oestrogen receptor binding ability of bixlozone.

The incorporated Toolbox Estrogen Receptor (ER) binding profiling scheme classifies chemicals as non-ER binders or binders depending on molecular weight (MW) and structural characteristics of the chemicals and is based on structural and parametric rules extracted from literature sources and supported by experimental data. For bixlozone the Toolbox ER binding profiling predicted the compound as a "non-binder, without OH or NH2 group".

The ER Expert System (ERES) Profiler (rtER Expert System ver.1 – USEPA) is an effects-based automated system available in the OECD QSAR Toolbox predicting oestrogen receptor binding affinity. The profiler consists of molecular definitions which mimic the structural criteria of chemical classes which are potential oestrogen receptor-binders covered by the US EPA ERES. No alert was found for bixlozone using this ED profiler.

Overall the OECD QSAR Toolbox predicted that bixlozone is not a ER-binder.

2. OECD CF 150 Level 2 – in Vitro Mechanistic Studies

Since bixlozone is a new active substance, it has not been evaluated as part of the US EPA ToxCAST or ESDP21 programmes. No OECD CF 150 Level 2 studies have been conducted for either mammalian or non-mammalian endpoints.

3. *OECD CF 150 Level 3 – in Vivo Mechanistic Studies*

No Level 3 studies have been conducted with bixlozone.

4. OECD CF 150 Level 4 Studies Assessing Mammalian Endocrine Sensitive Endpoints

Level 4 studies conducted between 2013 and 2017 included 28-day and 90-day oral repeated-dose toxicity studies in the rat, mouse and dog, a 12-month repeated-dose toxicity study in dog, a 28-day dermal repeated-dose toxicity studies in the rat, prenatal developmental toxicity studies in the rat and the rabbit, and long-term toxicity studies in the rat and mouse. All studies were conducted according to the respective OECD guidelines and all of them, with the exception of dose-ranging finding studies, were performed in accordance with GLP principles. The number of organs or tissues subject to evaluation (principally organ weight and/or histopathological assessment) depended on the study type conducted. The following organs were primarily considered for assessment in this context: liver, thyroid, pituitary, adrenal glands, and reproductive organs/tract (e.g., testes, ovaries and uterus).

Moreover a literature review was conducted in line with the recommendations of the EFSA guidance on the Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (EFSA Journal 2011;9(2):2092). No relevant studies were retrieved for bixlozone or any of its metabolites and more details can be found in Section B.6.10.

Data were populated in the Excel template provided as Appendix E of the EFSA/ECHA guidance (2018). According to this template each study was given a unique identification number (Study ID Matrix) that is important for its identification in the data-matrix and Lines of Evidence (LoE) spreadsheets on Excel (Data not shown, see Section B.6.8.3.2 Assessment of the evidence)

A summary of all Level 4 toxicology studies considered for bixlozone, including the Study ID Matrix is outlined in the table below:

Table B 6.8.3.1 : Outline of the dataset considered in the mammalian toxicology ED assessment - studies conducted with bixlozone

Type of studies	Study	Species	OECD Test Guideline No.	Reference
Repeated-dose toxicity studies Section **Error! Reference source not found.**	28-day oral toxicity study (diet)	Rat	407 (2008)	[redacted] (2015a) Study no. [redacted]-105108 **Study ID Matrix: 1**
	28-day oral toxicity study (diet)	Mouse	407 (2008)	[redacted] (2015b) Study no. [redacted]-105109 **Study ID Matrix: 2**
	28-day oral dose range-finding toxicity study (diet)	Dog	409 (1998)	[redacted] (2016b) Study no. [redacted]-105117 **Study ID Matrix: 3**
	90-day combined oral toxicity and neurotoxicity study (diet)	Rat	408 (1998) and 424 (1997)	[redacted] (2016a) Study no. [redacted]-105119 **Study ID Matrix: 4**
	90-day oral toxicity study (diet)	Mouse	408 (1998)	[redacted] (2016b) Study no. [redacted]-

Type of studies	Study	Species	OECD Test Guideline No.	Reference
				105118 **Study ID Matrix: 5**
	90-day oral toxicity study (capsule)	Dog	409 (1998)	[redacted] (2016c) Study no. [redacted]-105124 **Study ID Matrix: 6**
	12-months oral toxicity study (capsule)	Dog	452 (2009)	[redacted] (2017) Study no. [redacted]-105125 **Study ID Matrix: 9**
	28-day dermal toxicity study	Rat	410 (1981)	[redacted] (2016) Study no. [redacted]-105181 **Study ID Matrix: 10**
Long-term toxicity studies Section **Error! Reference source not found.**	Combined chronic toxicity/carcinogenicity study (diet)	Rat	OECD 453 (2009)	[redacted] (2017) Study no. [redacted]-105121 **Study ID Matrix: 7**
	Carcinogenicity study (diet)	Mouse	OECD 451 (2009)	[redacted] (2017) Study no. [redacted]-105120 **Study ID Matrix: 8**
Reproductive toxicity studies Section **Error! Reference source not found.**	Prenatal developmental toxicity range-finding study (gavage)	Rat	414 (2001)	[redacted] (2016d) Study no. [redacted]-105129 **Study ID Matrix: 11**
	Prenatal developmental toxicity study (gavage)	Rat	414 (2001)	[redacted] (2016e) Study no. [redacted]-105130 **Study ID Matrix: 12**
	Prenatal developmental toxicity range-finding study (gavage)	Rabbit	414 (2001)	[redacted] (2014a) Study no. [redacted]-105131 **Study ID Matrix: 13**
	Prenatal developmental toxicity study (gavage)	Rabbit	414 (2001)	[redacted] (2015) Study no. [redacted]-105132 **Study ID Matrix: 14**

5. *OECD CF 150 Level 5 – in Vivo Mammalian Assays providing more comprehensive data on Adverse Effects on Endocrine Relevant Endpoints over More Extensive Parts of Organism Life Cycles*

Level 5 studies were conducted between 2014 and 2016 according to the current OECD guideline and included a range-finding reproductive toxicity study and a main two-generation reproductive toxicity study; both studies were conducted in the rat.

The following organs were primarily considered for assessment in this context: liver, thyroid, pituitary, adrenal glands, and reproductive organs/tract (e.g., testes, ovaries and uterus).

A summary of all Level 5 studies considered for mammalian toxicology, including the Study ID Matrix is outlined in the table below:

Table B 6.8.3.2 : Outline of the Level 5 dataset considered in the mammalian toxicology ED assessment - studies conducted with bixlozone

Type of studies	Study	Species	OECD Test Guideline No.	Reference
Reproductive toxicity studies Section B.6.6	Two-generation range-finding reproductive toxicity (diet)	Rat	OECD 416 (2001) including landmarks of sexual maturation	[redacted] (2016b) Study no. [redacted]-105133 Study ID Matrix: 15
	Two-generation reproductive toxicity (diet)	Rat	OECD 416 (2001) including landmarks of sexual maturation	[redacted] (2016c) Study no. [redacted]-105134 Study ID Matrix: 16

B.6.8.3.2. ***Assessment of the evidence***

In this step, the information is assembled into lines of evidence, integrating information for both adversity and endocrine activity for the EATS modalities. The data were included in the Excel template provided as Appendix E of the EFSA/ECHA 2018 guidance (data not shown). According to this template each study was given a unique identification number (Study ID Matrix) that is important for its identification in the data-matrix and Lines of Evidence (LoE) spreadsheets of the Excel. The lines of evidence for adverse effects and endocrine activity related to the EATS modalities from *in vivo* studies were provided by the applicant and were reviewed by the UK (study report 2018WHP-ISX4276, revision N°2, data not shown).

The assessment of the evidence provided was divided into the following categories:

i) a review of the Estrogen, Androgen and Steroidogenic (EAS) modalities and

ii) a review or the Thyroid (T) modality.

All endocrine relevant parameters for which adverse changes were identified, were evaluated by HSE and are discussed below mainly to differentiate primary effects from those considered secondary to other toxic effects. Changes not considered being treatment-related or within the normal physiological range in the previous sections of the B6 have not been included in this assessment.

1. EAS modalities

Several parameters relevant to assessing the endocrine disrupting potential of bixlozone for the EAS modalities have been evaluated in a number of toxicology studies. These parameters include developmental effects, and effects on sexual/reproductive organs and performance in both Level 4 and Level 5 studies.

Male EAS-mediated parameters

Prostate findings (weight changes, histopathology) in rats and dogs

In the 2-generation reproductive toxicity study in the rat ([redacted], 2016c; ID Matrix: 16), mononuclear cell infiltration (chronic inflammation) of the prostate was evident at the top dose of 140 mg/kg bw/day in males of both generations, reaching statistical significance for the F_1 males. This effect occurred without any change in organ weight; however there was systemic toxicity observed in this treated group (decreased body weights and body weight gain, increased liver and kidney weights with associated histopathological findings), with the MTD (Maximum Tolerated Dose) being reached. Overall the prostate inflammation seen in the top dose males in both generations was considered treatment-related and adverse in this study but did not lead to any functional changes in fertility and reproductive performance in the same study. No such finding was noted in the other relevant rat studies (28-day, 90-day and 2-year rat studies) up to the highest dose tested at 750 mg/kg bw/day (28-day study), and there were no apparent changes in prostate weight noted in any of the available rat studies. Overall, considering the lack of consistency among different studies, the presence of systemic toxicity reaching the MTD and the lack of functional changes, these prostate findings in the rat are considered to be unspecific, of minimal toxicological significance and unlikely to be related to an endocrine mechanism.

In the dog, in the 90-day capsule study ([redacted], 2016c; ID Matrix: 16) a dose-dependent reduction in relative prostate weight (> 10 %) was observed from 100 mg/kg bw/day up to the top dose of 750 mg/kg bw/day. Histology features of immature prostate glands were also observed from 300 mg/kg bw/day and correlated well with the organ weight change observed for these animals. Given the absence of effects on body weight development in this study, the effects on the prostate were considered treatment-related and adverse from 300 mg/kg bw/day (dose at which histopathology correlate also occurred). However there were no such findings seen in the 1-year study up to 500 mg/kg bw/day and in the 28-day study. On this basis, the prostate findings reported in the dog were considered on balance to be of minimal toxicological significance and of no relevance to the reproductive performance of the dog. In addition, in isolation, they are unlikely to be related to an endocrine mechanism.

Decreased sperm in epididymes in the mouse

A slightly higher incidence of reduced epididymal sperm was seen in males at 126 and 647 mg/kg bw/day at terminal sacrifice in the 18-month mouse carcinogenicity study ([redacted], 2017; Study ID Matrix: 8). No significant systemic toxicity occurred at these doses. No other reproductive organs were affected in males. No such findings were seen in the 90-day mouse study up to the top dose of 930 mg/kg bw/day. It is most likely that these mild and isolated changes occurring during the reproductive senescence of the male mouse are of minimal toxicological significance and of no relevance to the reproductive performance of the mouse. Without effects on other reproductive organs, these epididymal changes are unlikely to be related to an endocrine mechanism.

Conclusion for male EAS-mediated parameters

There were some weight changes and / or histopathology findings observed in the prostate in the rat and the dog; however they were inconsistent across different studies. The findings reported in each species were isolated events not supported by any other relevant Level 4 studies. Moreover, the prostate findings of the Level 5 2-generation reproductive toxicity study in the rat were not accompanied with any functional impairment of spermatogenesis or reproduction and occurred in the presence of systemic toxicity; thus the finding does not indicate a specific adverse effect on the prostate in the rat. No other male reproductive organs were affected in the rat or dog.

A slightly higher incidence of reduced epididymal sperm was seen in the mouse in the 18-month carcinogenicity study. However, no other reproductive organs were affected and no such findings were seen in the 90-day mouse study.

No other relevant male EAS parameters were affected in the rat, mouse, dog or rabbit. Overall, there is no clear pattern of adversity for male EAS parameters and the reported changes on prostate in the rat and dog and on epididymis in the mouse are unlikely to be related to an endocrine mechanism.

Female EAS-mediated parameters

There are no treatment-related effects on female EAS-mediated parameters.

EAS-mediated reproductive and developmental parameters

No EAS-mediated reproductive and developmental effects were observed in either the F_0 or F_1 generations in the 2-generation rat study ([redacted], 2016c; Study ID Matrix: 16). Bixlozone had no effect on male or female fertility or reproductive performance up to the top dose of 140/187 mg/kg bw/day; gestation duration, oestrus cycle and spermatogenic endpoints were also unaffected by treatment. There was also no effect on litter size, sex ratio, pup survival and developmental landmarks.

The age of attainment of vaginal opening of F1 pups was statistically significantly greater at the top dose (187 mg/kg bw/day) compared to the corresponding controls (33.6 days compared with 31.7 days). The mean body weights of the female pups at the age of attainment were unaffected by treatment with bixlozone, which indicates that the delay in vaginal patency was the consequence of reduced pup body weight development, because, once the pup body weight was similar to that of the controls, vaginal opening was attained. Moreover the values seen at the top-dose were well within the laboratory HCD provided, although these cover a period of 10 years. In addition, there were no notable effects on other developmental landmarks and these females went on to mate successfully and produce the F2 generation. Overall, HSE considers this finding the secondary consequence of reduced post-weaning female pup body weight development and not a specific endocrine effect of bixlozone.

In both main (and range-finding) rat and rabbit prenatal developmental studies ([redacted], 2016c; Study ID Matrix: 12 & [redacted], 2015; Study ID Matrix: 14), gravid uterine weights were unaffected at all dose levels.

It is noted that the updated OECD guideline 414 (2018) for the 2-generation reproduction toxicity study requires the assessment of foetal anogenital distance as a developmental endpoint. This was not measured in the study conducted with bixlozone as it was commissioned before the updated guideline. However, the measurement of offspring anogenital distance was not triggered in this instance, as sex ratio and sexual maturity were not specifically affected by treatment in the study.

Adrenals - weight and histopathology

In the available repeated-dose toxicity studies there was one occurrence of higher relative adrenal weights described in the 28-day range-finding oral (diet) toxicity study in dogs ([redacted], 2016b; Study ID Matrix: 3) at the top dose in both sexes (1015 / 1110 mg/kg bw/day). However a clear dose-response was not evident and only 2 animals / sex were used in this range-finding study; there were also no histologic correlates and findings were compatible with adaptive changes associated with a stress response owing to the severe loss in body weight gain and food consumption observed at the top dose. There were no similar findings reported in the 90-day and 12-month dog studies using capsules as the method of oral administration ([redacted], 2016c; Study ID Matrix: 6 & [redacted], 2017; Study ID Matrix: 9), which indicates that the organ changes seen in the 28-day study were most likely the secondary consequence of the general toxicity caused by the unpalatability of the diet.

There were no treatment-related changes in adrenal weights and/or histopathology reported in any of the rat, mouse and rabbit studies available for bixlozone.

Overall repeated exposure to bixlozone in the rat, mouse, dog and rabbit was not associated with any clear treatment-related effects on the adrenal gland.

a. Overall conclusion on adverse effects related to EAS-modalities

In all species investigated (rat, mouse, dog) there were no specific adverse effects on reproductive organs and other endocrine organs related to EAS modalities (e.g. adrenal, pituitary, mammary – please refer to Section **Error! Reference source not found.**) following repeated exposure to bixlozone. In addition, there were no specific adverse effects on reproduction in the rat and on development in the rat and the rabbit.

Overall, there was no clear pattern of adversity for the EAS modalities.

2. T-modality

Lines of evidence for adverse effects related to T-modality from *in vivo* studies were supplied by the applicant (data not shown).

Thyroid weight changes

In the 90-day dog oral (capsule) study ([redacted], 2016c; Study ID Matrix: 6) a dose-related increase in the thyroid/parathyroid weights was reported across all dose-groups (from 30 mg/kg bw/day) in both sexes, which at the top dose (750 mg/kg bw/day) was 43 % (relative) and 42.5 % (absolute) larger than controls in males and 25 % (relative) and 30 % (absolute) larger in females; the results were variable since at no point statistical significance was reached. No effects on body weight, body weight gain or food consumption were noted in this study. Histopathology examination did not correlate with the above organ weight findings; however the magnitude of the weight increase seen at the top dose in females and from 300 mg/kg bw/day in males justified adversity within the study. The thyroid was however not affected in the 28-day study up to the top dose of approx. 1340/1080 mg/kg bw/day (M/F) or in the 1-year study up to 500 mg/kg bw/day. Therefore it is most likely the changes in thyroid weight seen in the 90-day dog study were a spurious finding.

There were no biologically relevant thyroid weight changes noted in the rat and the mouse in any of the relevant studies investigating potential adverse effects on the thyroid.

Histopathology – Non neoplastic and neoplastic findings

In the 90-day repeated dose toxicity study in the rat ([redacted], 2016a; Study ID Matrix: 4), there was an increased incidence of mild follicular cell hypertrophy noted at the top dose in males (3/10 at 505 mg/kg bw/day) and females (5/10 at 351 mg/kg bw/day) that was absent in concurrent controls. The hypertrophy was not observed at the end of the 28-day recovery period. Thyroid weights and circulating levels of thyroid hormones were not measured in the study to confirm the biologically relevance of the finding. It was noted that the thyroid effects occurred concomitantly with systemic toxicity in excess of the MTD. This was characterised by one death, statistically significantly lower body weights and body weight gains and clear adverse liver effects (absolute and relative liver weights statistically significantly increased and > 15 % in both sexes, hepatocellular hypertrophy in both sexes with vacuolation in males, relative liver weights remained greater than controls in both sexes after the recovery period). Moreover there were no other occurrences of thyroid follicular cell hypertrophy reported in any other rat studies (28-day, 2-generation reproduction toxicity and 2-year studies) and in the mouse, the dog and the rabbit, including in studies where comparable/higher dose levels of bixlozone were tested.

Thus the relationship of this isolated histopathology observation to a specific effect of bixlozone on the thyroid is considered to be unlikely.

In the carcinogenicity study in rats ([redacted], 2017; Study ID Matrix: 7), there was a non-statistically significant but dose-related increase in the incidence of follicular cell adenomas (benign tumours; 2/60 (3.3 % incidence) vs 0/60 in controls) and of the follicular cell carcinoma (1/60 (1.7 % incidence) vs 0/60 in controls) in the thyroid gland of females at the top-dose of 3000 ppm (167 mg/kg bw/day) in comparison to controls.

The HCD provided for these tumour findings are not fully compliant with the data requirements laid out in Reg 283/2013 (section 5, point 3) because they cover more than 5 years, however they are from the same laboratory and strain of rat and derived from a reasonable number of studies; thus, they can be considered by HSE in a WoE approach. These significantly extended HCD (date range 1999 – 2017) show that at the top dose both tumour incidences are slightly higher than the mean % incidence but well within the HCD range. The applicant also provided the maximum control numerical and % incidence values derived from studies conducted between 2009 and 2017 i.e. performed around the date (2014) of the current study: the maximum numerical incidence was 3 (4.7 % incidence) for follicular cell adenoma and 1 (1.7 %) for follicular cell carcinoma. Thus the thyroid tumour incidences seen in the study in females at the top dose are consistent with the top of the range of the control groups monitored around the time of the study.

The finding is sex specific since no relevant or dose-related increase in incidence was observed in males. Moreover, it is noted that the incidence of follicular cell adenoma (benign) in the control male group is similar (3.4 %) to the incidence seen in the top dose female group (3.3 %) and that the incidence of follicular cell carcinoma was for both sexes very low (maximum of 1 case per 50 / 60 rats per group) and clearly not dose-related.

The available kinetics data appear to support the sex specificity of the response since in rats the females show a higher systemic exposure than males. However, a tissue distribution study showed that less than 0.1 % of the administered dose was found in the thyroid in both sexes following oral administration of bixlozone, with the thyroid of males being more exposed than females' following a single high oral dose (500 mg/kg bw) or a repeated low oral dose (5 mg/kg bw/day, 14 days).

Considering the biological plausibility of the finding, it is noted that there were no other associated findings noted in the thyroid (e.g. hyperplasia, hypertrophy) in the study to support the tumorigenic response, even though the thyroid was identified as a target organ of toxicity in the 90-day rat study. Yet, the histopathology changes in the 90-day study were seen at doses higher than the top dose tested in this carcinogenicity study, with mild follicular cell hypertrophy observed in both sexes at the top dose of 505 / 351 mg/kg bw/day only and without associated changes in the thyroid weights. There were no histopathology findings seen at the lower doses of 121 / 150 mg/kg bw/day. Therefore, the biological plausibility of these thyroid tumour findings appears to be low.

The applicant submitted a review of the incidence of these thyroid tumours by a PWG panel. The PWG confirmed the incidences determined by the study pathologist. Therefore, these data have not been considered further by HSE.

Overall, considering the sex specificity of the response, the low incidence of the tumours and the low biological plausibility of the finding, HSE conclude that the thyroid tumours observed in female rats at the top dose are chance findings unrelated to treatment.

Overall conclusion on adverse effects related to the T-modality

Overall, it was shown that repeated exposure to bixlozone in Level 5 and Level 4 studies in rats, mice and dogs was not associated with any clear or specific effects on the thyroid gland, with only isolated incidences of thyroid weight changes reported in the 90-day dog study or histopathology described in the 90-day rat study. Therefore there is no evidence of a clear pattern of adversity for the T modality.

In addition, there is no indication of adverse pre- and post-natal neurological development of the offspring in the available Level 5 2-generation reproduction toxicity study in the rat ([redacted], 2016c; Study ID Matrix: 16) and the Level 4 developmental toxicity studies in the rat and the rabbit ([redacted], 2016e; Study ID Matrix: 12 & [redacted], 2015; Study ID Matrix: 14). Therefore a potential concern for neurodevelopment is considered unlikely for bixlozone.

Overall bixlozone does not present a clear pattern of adversity for the T modality in relation to effects on the thyroid gland and/or neurodevelopment effects.

B.6.8.3.3. *Conclusion*

1. Conclusion and sufficiency of the data

The data package available to characterise the toxicity profile of bixlozone is considered sufficient to determine whether EATS-mediated adversity is exerted following repeated exposure to bixlozone. All studies from the data package presented in the DAR were conducted recently (2015 – 2017) and followed the most current OECD guidelines available at the time of study and were compliant with GLP standards, with the exception for the dose range-finding experiments (but this is acceptable).

EAS modalities

EAS-mediated adversity has been sufficiently investigated, based on a modern 2-generation reproduction toxicity study conducted by [redacted] (2016c; Study ID Matrix: 16); the study was fully compliant with the OECD Guideline No. 416 (2001) and followed GLP standards.

In all species investigated (rat, mouse, dog) there were no specific adverse effects on reproductive organs and other endocrine organs related to EAS modalities (e.g. adrenal, pituitary, mammary – please refer to Section **Error! Reference source not found.**) following repeated exposure to bixlozone. In addition, there were no specific adverse effects on reproduction in the rat and on development in the rat and the rabbit. Overall, there was no clear pattern of adversity for the EAS modalities.

No EAS activity studies are available for bixlozone and none are necessary. However, bixlozone was predicted not to bind to ER receptors using (Q)SAR profiling.

Conclusion of the assessment of EAS-modalities

Based on scenario 1a of the ECHA/EFSA/JRC guidance (2018) for the identification of endocrine disruptors in the context of Regulations (EU) 528/2012 and (EC) No 1107/2009, HSE concludes that bixlozone does not meet the ED criteria for the EAS modalities and that these modalities have been sufficiently investigated for this compound.

Analysis of the evidence and identification of relevant scenario for the ED assessment of EATS-modality - Selection of relevant scenario

Adversity based on EAS-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected
No (sufficiently investigated)	Yes/**No**	**1a**	Conclude: ED criteria not met because there is no **'EAS-mediated'** adversity	X
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no EAS-mediated endocrine activity observed	
No (not sufficiently investigated)	No (not sufficiently investigated)	2a (iii)	Generate missing level 2 and 3 information. Alternatively, generate missing "EATS-mediated" parameters. Depending on the outcome move to corresponding scenario	
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

T modality

T-mediated adversity (thyroid weight and histopathology) has been sufficiently investigated, based on the following studies in which thyroid effects were investigated:

- 28-day oral toxicity studies in the rat, mouse, dog (OECD TG No. 407)
- 90-day oral toxicity studies in the rat, mouse, dog (OECD TG No. 408)
- Chronic toxicity / carcinogenicity studies in the rat and mouse (OECD TG No. 453)
- 2-generation reproduction toxicity study in the rat (OECD TG No. 416).

All were modern OECD compliant studies; however they were completed before the requirement to investigate additional thyroid-related parameters was added to OECD Guideline 408 (2018) (28-day study) and 414 (2018) (pre-natal developmental study). Overall, the available data showed that bixlozone does not present a clear pattern of adversity for the T modality in relation to effects on the thyroid gland and/or neurodevelopment effects.

No thyroid activity studies are available for bixlozone and none are necessary.

Conclusion of the assessment of T-modality

Based on scenario 1a of the ECHA/EFSA/JRC guidance (2018) for the identification of endocrine disruptors in the context of Regulations (EU) 528/2012 and (EC) No 1107/2009, it is possible to conclude that bixlozone does not meet the ED criteria for the T modality and that this has been sufficiently investigated.

Analysis of the evidence and identification of relevant scenario for the ED assessment of T-modality - Selection of relevant scenario

Adversity based on T-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected
No (sufficiently investigated)	**Yes/No**	**1a**	**Conclude: ED criteria not met because there is no 'T-mediated' adversity**	**X**
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no EAS-mediated endocrine activity observed	
No (not sufficiently investigated)	No (not sufficiently investigated)	2a (iii)	Generate missing level 2 and 3 information. Alternatively, generate missing "EATS-mediated" parameters. Depending on the outcome move to corresponding scenario	
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

B.6.8.3.4. *Overall conclusion on the ED assessment for humans*

Overall, based on this analysis, bixlozone does not meet the ED criteria of Regulation (EC) No 2018/605 of 19 April 2018, amending Annex II to Regulation (EC) No 1107/2009.

HSE concludes that for the EATS-modalities bixlozone is not an ED and its ED potential has been sufficiently investigated and that no further information is required.

B.6.9. MEDICAL DATA AND INFORMATION

B.6.9.1. Medical surveillance on manufacturing plant personnel and monitoring studies

According to data maintained by FMC Corporation, it is estimated that more than 1,200 workers were involved in the research and development of bixlozone Regarding the synthesis, manufacture, and analytical processes of bixlozone and its formulations, it is estimated that approximately 250 workers were involved. In Brazil, an estimated 70 workers were potentially exposed to some extent to bixlozone during the biological and regulatory field tests. Regarding global research and development for regulatory studies, approximately 20 separate Contract Research Organizations have been involved with an estimated 200 personnel having been potentially exposed to bixlozone. Lastly, regarding global field development, more than 700 people were expected to be exposed to the compound.

As of 11 January 2018, there were no reports of diseases or adverse health effects attributed to exposure associated with the handling, testing or manufacture of bixlozone and formulations containing bixlozone. At the time of submission of the bixlozone dossier, there were no reports of clinical cases and poisoning.

B.6.9.2. Data collected on humans

Bixlozone has not been introduced in the market yet, therefore there is no information on record.

B.6.9.3. Direct observation

Bixlozone has not been introduced in the market yet, therefore there is no information on record.

B.6.9.4. Epidemiological studies

Bixlozone has not been introduced in the market yet, therefore there is no information on record.

B.6.9.5. Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical test

Bixlozone is not considered to be acutely toxic *via* the oral and the dermal route but is proposed to be classified for acute inhalation toxicity Category 4 based on read-across to the closely related active substance clomazone. It is not a skin or eye irritant, or a skin sensitiser.

In animal studies, clinical signs of toxicity (reduced body weight / body weight gain, adverse effects on liver and kidneys) were evident at approximately 140 mg/kg bw/day and above. The same would be expected to occur in humans if these kind of dose levels were consumed. However, no cases of intoxication with bixlozone have yet been observed.

No specific clinical tests have been performed in humans.

B.6.9.6. Proposed treatment: first aid measures, antidotes, medical treatment

General	Terminate exposure, remove person from scene of spillage or other contamination
In case of skin contact:	Remove contaminated clothing and thoroughly wash the affected parts of the body with soap and water
In case of eye contact:	Rinse eyes with clean water for several minutes. Obtain medical advice
In case of ingestion:	Do not induce vomiting. Obtain medical advice

No antidote is known, apply symptomatic treatment.

In case of skin/eye contact	Decontamination
In case of ingestion	If the amount ingested is judged less than a potentially toxic dose, employ general supportive measures. Administration of activated charcoal and a purgative in a large quantity of water is indicated even in cases when considerable time has elapsed because excretion via the bile is significant.

B.6.10. REFERENCES RELIED ON

Literature review

This literature review was conducted by the applicant following the current EFSA Guidance (EFSA Journal 2011;9(2):2092) for identifying scientific peer-reviewed open literature on the active substance F9600 and its relevant metabolites, which may affect the assessment of human health, animal health and/or the environment, as required by the data requirements of Regulation (EC) No 1107/20091.

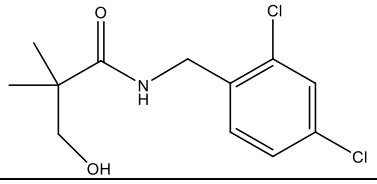
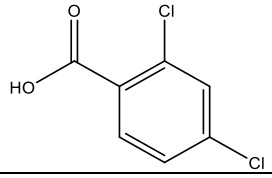
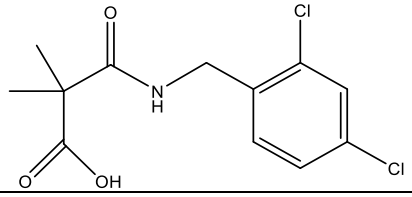
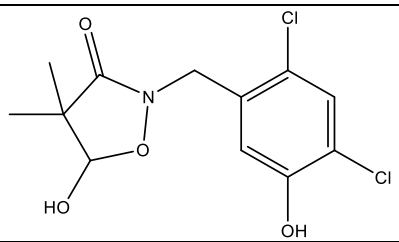
Study	LITERATURE REVIEW REPORT
Reference	Exponent International Ltd
Date performed	15 February 2018
Test facility	Exponent International Ltd – U.K.
Report reference	1508442.UK0 – 5012
Guideline(s)	EFSA Journal 2011;9(2):2092. Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009.
Deviations from the guideline	The applicant did not consider full-text publications in their Step 2 of relevance assessment, only abstracts. HSE requested the full text assessment of one study found to be relevant for toxicology, which can be found in the results section below.
GLP	N/A
Date of the search:	15 February 2018
Date span of the search:	2008 to 2018
Study acceptable	Yes

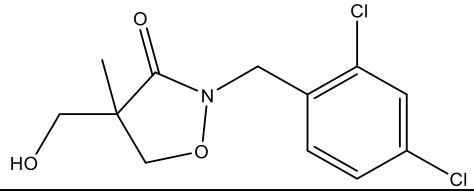
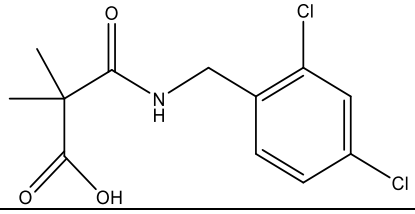
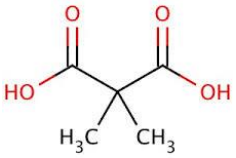
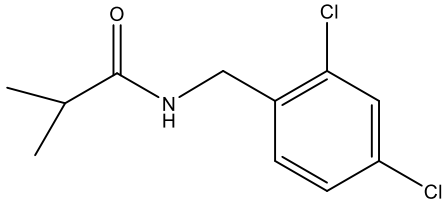
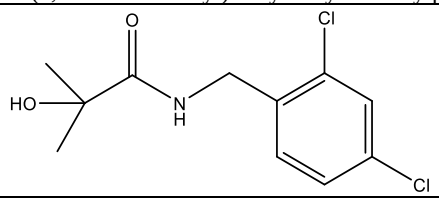
Methods

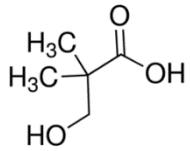
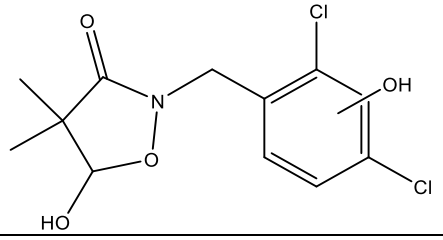
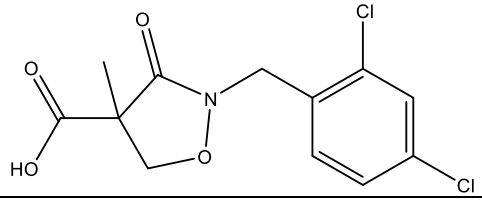
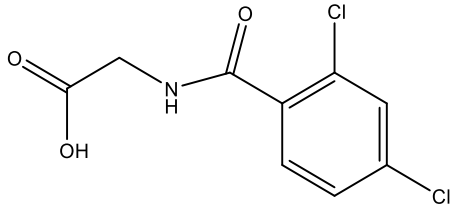
Key words

F9600 and the following metabolites were considered for the literature search:

Description/justification of search terms	Search terms	Search source
Active substance		
ISO common names of active substance	Not available	Dialog
Active substance synonyms	F9600	Dialog
Chemical name (CA):	2-[(2,4-dichlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone	Dialog
Chemical name (IUPAC):	2-[(2,4-dichlorophenyl)methyl]-4,4-dimethyl-1,2-oxazolidin-3-one	Dialog
CAS numbers	81777-95-9	STN
Development Codes:	F9600	Dialog
CIPAC No.	Not assigned	
Metabolite 1		
Code:	3-hydroxypropanamide-F9600	Dialog
Chemical name (IUPAC):	N-(2,4-dichlorobenzyl)-3-hydroxy-2,2-dimethylpropanamide	Dialog

Description/justification of search terms	Search terms	Search source
Structure		
CAS numbers	Not assigned	
Metabolite 2		
Code:	2,4-Dichlorobenzoic acid	Dialog
Chemical name (IUPAC):	2,4-dichlorobenzoic acid	Dialog
Structure		
CAS numbers	50-84-0	STN
Metabolite 3*		
Code:	F9600 Dimethyl Malonamide	Dialog
Chemical name (IUPAC):	3-((2,4-dichlorobenzyl)amino)-2,2-dimethyl-3-oxopropanoic acid	Dialog
Structure		
CAS numbers	Not assigned	
Metabolite 4		
Code:	5-Hydroxy-F9600	Dialog
Chemical name (IUPAC):	2-(2,4-dichlorobenzyl)-5-hydroxy-4,4-dimethylisoxazolidin-3-one	Dialog
Structure		
CAS numbers	Not assigned	
Metabolite 5		
Code:	5'-Hydroxy-F9600	Dialog
Chemical name (IUPAC):	2-(2,4-dichloro-5-hydroxybenzyl)-4,4-dimethylisoxazolidin-3-one	Dialog
Structure		
CAS numbers	Not assigned	
Metabolite 6		
Code:	6'-Hydroxy-F9600	Dialog
Chemical name (IUPAC):	2-(2,4-dichloro-6-hydroxybenzyl)-4,4-dimethylisoxazolidin-3-one	Dialog
Structure		
CAS numbers	Not assigned	
Metabolite 7		

Description/justification of search terms	Search terms	Search source
Code:	4-Hydroxy-methyl-F9600	Dialog
Chemical name (IUPAC):	2-(2,4-dichlorobenzyl)-4-(hydroxymethyl)-4-methylisoxazolidin-3-one	Dialog
Structure		
CAS numbers	Not assigned	
Metabolite 8*		
Code:	Dimethyl malonamide- F9600	Dialog
Chemical name (IUPAC):	3-((2,4-dichlorobenzyl)amino)-2,2-dimethyl-3-oxopropanoic acid	Dialog
Structure		
CAS numbers	Not assigned	
Metabolite 9		
Code:	Dimethyl malonic acid	Dialog
Chemical name (IUPAC):	2,2-Dimethylmalonic acid	Dialog
Structure		
CAS numbers	595-46-0	STN
Metabolite 10		
Code:	F9600-isobutyramide	Dialog
Chemical name (IUPAC):	N-(2,4-dichlorobenzyl)isobutyramide	Dialog
Structure:		
CAS numbers	Not assigned	
Metabolite 11		
Code:	Hydroxy-Isobutyramide	Dialog
Chemical name (IUPAC):	N-(2,4-dichlorobenzyl)-2-hydroxy-2-methylpropanamide	Dialog
Structure		
CAS numbers	Not assigned	

Description/justification of search terms	Search terms	Search source
Metabolite 12		
Code:	3-hydroxypivalic acid	Dialog
Chemical name (IUPAC):	2,2-Dimethyl-3-hydroxy propionic acid	Dialog
Structure		
CAS numbers	4835-90-9	STN
Metabolite 13**		
Code:	5-OH, 5'-OH Di-Hydroxy-F9600	Dialog
Chemical name (IUPAC):	2-(2,4-dichloro-5-hydroxy benzyl)-5-hydroxy-4,4-dimethylisoxazolidin-3-one	Dialog
Structure		
CAS numbers	Not assigned	
Metabolite 13**		
Code:	4-carboxy-F9600	Dialog
Chemical name (IUPAC):	2-(2,4-dichlorobenzyl)-4-methyl-3-oxoisoxazolidine-4-carboxylic acid	Dialog
Structure		
CAS numbers		
Metabolite 14		
Code:	2,4-Dichloroippuric acid	Dialog
Chemical name (IUPAC):	N-(2,4-dichlorobenzoyl)glycine	Dialog
Structure		
CAS numbers	Not assigned (2,5 analogue has CAS number)	

HSE identified two errors in this table detailing the metabolites included in the database searches however they did not confer any deficiency in the search strategy conducted by the applicant:
* Metabolites 3 and 8 are identical
** Two different metabolites have been assigned as metabolite 13

Since F9600 is a new active substance and no formulations have yet been commercialised, the search did not include any product names.

Search strategy

The search strategy was based on a single-concept search in both STN and Dialog databases. Patents and conference papers were excluded as these were not expected to contain information that was both relevant and reliable.

STN Databases is an online database service that provides global access to published research, journal literature, patents, structures, sequences, properties, and other data. As a neutral platform STN provides access to a broad range of databases from the most renowned database producers worldwide. STN is operated jointly by Chemical Abstracts Service (CAS) and FIZ Karlsruhe worldwide and is represented in Japan by the Japan Association for International Chemical Information (JAICI).

STN DATABASES *http://www.stn-international.de/database_list.html?&no_cache=1&cHash=*

STN-DATABASES:	**Frequency of updates**
ANABSTR (Analytical abstracts)	Updated weekly
BIOSIS (BIOSIS PREVIEWS®)	Updated weekly
CABA (CA Abstracts)	Updated weekly
CAplus (Chemical abstracts plus)	Updated daily
CAplus (Toxicology focus)	Updated daily
CAplus (Analytical chemistry focus)	Updated daily
Chemical Abstracts REGISTRY	Updated daily
EMBASE (Excerpta Medica)	Updated daily
ESBIOBASE (Elsevier Current Research in Biology and BioScience)	Updated weekly
MEDLINE	Updated daily
NAPRALERT (Natural Products Alert)	Occasional updates
RTECS (Registry of Toxic Effects of Chemical Substances)	Updated quarterly
SCISEARCH (Science Citation Index)	Updated weekly
TOXCENTER (Toxicology Center produced by American Chemical Society CAS)	Updated weekly

Dialog is the premier online retrieval service with the most comprehensive content collection and most powerful search language available. Dialog is the worldwide leader in providing online-based information in science. The database holds data from more than 800 million unique records of key information, accessible via the Internet. Content areas include, but are not limited to, biomedical research, biotechnology, chemicals, environment, food and agriculture, medicine, and science and technology.

DIALOG DATABASES:	**Frequency of updates**
AGRIS	All PROQUEST databases are current and updated regularly (individual database is updated either daily, weekly or monthly)
Aqualine	
Aquatic Science & Fisheries Abstracts (ASFA)	
Chemical Engineering and Biotechnology Abstracts	
CSA Life Sciences Abstracts	
Ecology Abstracts	
ENVIROLINE®	
Environment Abstracts	
FSTA®	
FOODLINE®: Science	
GeoArchive	
GEOBASE(TM)	
MEDLINE®	
Meteorology & Geoastrophysical Abstracts.	
Pollution Abstracts	
ToxFile	
Toxicology Abstracts	
TOXLINE	
Water Resources Abstracts	

Overview of the search process

An overview of the search process for scientific peer-reviewed open literature in bibliographic databases is presented in the table below:

	STN Toxicology Database Cluster	**Dialog**
Date of the search:	15 February 2018	15 February 2018
Date span of the search:	2008 to 2018	2008 to 2018
Search strategies	Search Question: RN: 81777-95-9 RN: 81777-95-9 RN: 50-84-0 RN: 595-46-0 RN: 4835-90-9 NOT Document Type: conference NOT Document Type: patent	("2-[(2,4-dichlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone" OR "2-[(2,4-dichlorophenyl)methyl]-4,4-dimethyl-1,2-oxazolidin-3-one" OR "F9600" OR "3-hydroxypropanamide-F960" OR "N-(2,4-dichlorobenzyl)-3-hydroxy-2,2-dimethylpropanamide" OR "2,4-dichlorobenzoic acid" OR "F9600 Dimethyl Malonamide" OR "3-((2,4-dichlorobenzyl)amino)-2,2-dimethyl-3-oxopropanoic acid" OR "5-Hydroxy-F9600" OR "2-(2,4-dichlorobenzyl)-5-hydroxy-4,4-dimethylisoxazolidin-3-one" OR "5'-Hydroxy-F9600" OR "2-(2,4-dichloro-5-hydroxy benzyl)-4,4-dimethylisoxazolidin-3-one" OR "6'-Hydroxy-F9600" OR "2-(2,4-dichloro-6-hydroxy benzyl)-4,4-dimethylisoxazolidin-3-one" OR "4-Hydroxy-methyl-F9600" OR "2-(2,4-dichlorobenzyl)-4-(hydroxymethyl)-4-methylisoxazolidin-3-one" OR "Dimethyl malonamide- F9600" OR "Dimethyl malonic acid" OR "F9600-isobutyramide" OR "N-(2,4-dichlorobenzyl)isobutyramide" OR "Hydroxy-Isobutyramide" OR "N-(2,4-dichlorobenzyl)-2-hydroxy-2-methylpropanamide" OR "2,2-Dimethyl-3-hydroxy propionic acid" OR "5-OH, 5'-OH Di-Hydroxy-F9600" OR "2-(2,4-dichloro-5-hydroxy benzyl)- 5-hydroxy-4,4-dimethylisoxazolidin-3-one" OR "4-carboxy-F9600" OR "2-(2,4-dichlorobenzyl)-4-methyl-3-oxoisoxazolidine-4-carboxylic acid" OR "2,4-Dichloroippuric acid" OR "N-(2,4-dichlorobenzoyl)glycine" OR "3-Isoxazolidinone, 2-[(2,4-dichlorophenyl)methyl]-4,4-dimethyl-" OR "Benzoic acid, 2,4-dichloro-" OR "Propanedioic acid, 2,2-dimethyl-" OR "2,2-Dimethylpropanedioic acid" OR "Malonic acid, dimethyl-" OR "Propanedioic acid, dimethyl-" OR "2,2-Propanedicarboxylic acid" OR "Dimethylmalonic acid" OR "Dimethylpropanedioic acid" OR "Propanoic acid, 3-hydroxy-2,2-dimethyl-, methyl ester" OR "Hydracrylic acid, 2,2-dimethyl-, methyl ester" OR "2,2-Dimethyl-3-hydroxypropanoic acid methyl ester" OR "2-Methoxycarbonyl-2-

	STN Toxicology Database Cluster	Dialog
		methylpropan-1-ol" OR "3-Hydroxy-2,2-dimethylpropanoic acid methyl ester" OR "3-Hydroxy-2,2-dimethylpropionic acid methyl ester" OR "Hydroxypivalic acid methyl ester" OR "Methyl .beta.-hydroxypivalate" OR "Methyl 2,2-dimethyl-3-hydroxypropanoate" OR "Methyl 2,2-dimethyl-3-hydroxypropionate" OR "Methyl 3-hydroxy-2,2-dimethylpropanoate" OR "Methyl 3-hydroxy-2,2-dimethylpropionate" OR "Methyl hydroxypivalate") AND (at.exact("Article" OR "Book" OR "Book Chapter" OR "Government & Official Document" OR "Case Study" OR "Technical Report" OR "Report"))

Time period

The literature search has been performed to cover the 10 years prior to the expected submission of the dossier for the new active substance F9600, which was expected to be by June 2018.

Selection process

The selection process resulted in two categories of publication:

1. Studies considered being non-relevant after initial (rapid) review.
2. Potentially relevant articles requiring more detailed consideration of abstracts and / or full-text documents to assess relevance.

Criteria of relevance applied

Studies relevant to the dossier are those that inform one or more data requirement(s), including hazard identification, hazard characterisation and exposure assessment, for the active substance under assessment, its relevant metabolites, or plant protection products.

As part of the determination of relevancy for toxicology, the following criteria are considered to be fundamental when considering the relevance of an open-literature study (EFSA Journal 2011;9(2):2092):

- the test species,
- the test material,
- the use of different doses,
- the specific endpoints of interest.

Studies that are relevant to the data requirements are studies that appropriately address these components, i.e. studies which present a well-identified test material (including purity and impurity profile); a test relevant to the mammalian toxicological assessment (preferred species are rats and mice; the dog is the preferred non-rodent species); a number of animals per group sufficient to establish a statistical significance; several dose levels tested (at least 3), preferably including a negative control, to establish a dose response; relevant route of administration in terms of risk assessment (oral, dermal or inhalation); and a description of the observations, examinations and analysis or necropsy performed.

Data requirement (data point)	Criteria considered for relevance
Active substance	
Studies on absorption, distribution, metabolism and excretion in mammals (Section B.6.1)	1. Well-defined test material. 2. *In vivo* tests in relevant test species. 3. *In vitro* tests. 4. PBPK modelling. 5. Specific endpoint can be clearly related to this data requirement.
Acute toxicity (Section B6.2)	1. Well-defined test material. 2. Relevant test species. 3. Relevant route of exposure. 4. Specific endpoint can be clearly related to this data requirement.
Short-term toxicity (Section B6.3)	1. Well-defined test material. 2. Relevant test species. 3. Relevant route of exposure. 4. Specific endpoint can be clearly related to this data requirement.
Genotoxicity (Section B6.4)	1. Well-defined test material. 2. *In vitro* tests. 3. *In vivo* tests in relevant test species. 4. Specific endpoint can be clearly related to this data requirement.
Long-term toxicity and carcinogenicity (Section B6.5)	1. Well-defined test material. 2. Relevant test species. 3. Relevant route of exposure. 4. Specific endpoint can be clearly related to this data requirement
Reproductive toxicity (Section B6.6)	1. Well-defined test material. 2. Relevant test species. 3. Relevant route of exposure. 4. Specific endpoint can be clearly related to this data requirement.
Neurotoxicity studies (Section B6.7)	1. Well-defined test material. 2. In vivo tests in relevant test species. 3. Relevant route of exposure. 4. Specific endpoint can be clearly related to this data requirement.
Other toxicological studies (Section B6.8)	1. Well-defined test material. 2. In vitro tests. 3. In vivo tests in relevant test species. 4. Relevant route of exposure. 5. Specific endpoint can be clearly related to this data requirement.
Medical data (Section B6.9)	1. Well-defined test material. 2. Epidemiological studies. 3. Poisonings, clinical cases. 4. Relevant route of exposure.
Plant protection products	
Acute toxicity (Section B.6.1 (PPP))	1. Well-defined test material. 2. Relevant test species. 3. Relevant route of exposure. 4. Specific endpoint can be clearly related to this data requirement.
Data on exposure (Section B.6.4 (PPP))	1. Well-defined test material. 2. Field studies. 3. Calculations. 4. Specific endpoint can be clearly related to this data requirement.
Dermal absorption (Section B.6.2 (PPP))	1. Well-defined test material. 2. *In vitro* tests. 3. *In vivo* tests in relevant test species. 4. Specific endpoint can be clearly related to this data requirement.

Assessment of studies for relevance was carried out by reference to their titles and if necessary abstracts. Those studies that were considered to meet the relevance criteria, following review of their abstracts were obtained. The applicant further state that the full-text of these documents was assessed further to determine whether the information contained in the study could impact on the endpoints and risk assessment parameters related to the active substance.

HSE considered the selection process and noticed that it did not fully align with the EFSA recommendations cited above. Nine publications were found to be potentially relevant or of unclear relevance in the rapid assessment (using their titles and/or abstracts). In the second step, their relevance was again decided using the abstracts only, whilst the EFSA Guidance recommends that "full-text documents should be obtained for those summary records not excluded in step 1 and assessed in detail for their relevance". HSE has found only one publication by Svobodová *et al*., 2009 to be relevant to toxicology and considered that a full-text assessment of this article was a more appropriate approach to decide on its relevance. Therefore the applicant was asked to provide the full text assessment for this article and a robust justification as to why this article should be considered relevant or not for Toxicology.

Reviews of the relevance and reliability of the articles brought up in the literature search were carried out by experts in the relevant technical disciplines.

The reliability assessment for relevant studies was carried out according to Klimisch *et al.* (1997).

Code	Category
1	Reliable without restriction
2	Reliable with restriction
3	Not reliable
4	Not assignable

Results

Findings of the literature review

Summary of the review	**n**	**Consideration by HSE**
Total number of summary records retrieved after removing duplicates from all database searches	37	13 were retrieved using the STN Toxicology Database Cluster 24 were retrieved using Dialog
Number of summary records excluded after rapid assessment for relevance (by title/abstract)	28	None of the records excluded after rapid assessment for relevance were relevant to Toxicology.
Number of summary records of potential/unclear relevance assessed in further detail (by abstract/full-text)	9	Article 9 is of interest to Toxicology.
Number of studies excluded from further consideration after detailed assessment for relevance (by abstract/full-text)	9	
Number of studies not excluded for relevance after detailed assessment (i.e. relevant studies and studies of unclear relevance)	0	
Number of relevant and reliable studies (Klimisch criteria 1-2) identified by the literature search and appraisal process	0	

Thirty seven articles were retrieved after removing the duplicates from all database searches. After a rapid assessment for relevance using the title or abstract, 28 articles were removed from the selected articles and the 9 remaining articles of potential relevance to the regulatory data package for the active substance were assessed in further detail by examining the abstracts. For the articles considered to meet the criteria for relevance, an

assessment of the reliability of the study was carried out based on the approach described in Klimisch et al., (1997). HSE has found only one publication by Svobodová *et al*., 2009 to be relevant to toxicology; the following article was thus provided by the applicant and accompanied with an assessment of its relevance following the criteria of relevance above.

Information on the article provided

Svobodová K., Plačková M., Novotná V.and Cajthaml T. **"Oestrogenic and androgenic activity of PCBs, their chlorinated metabolites and other endocrine disruptors estimated with two *in vitro* yeast assays."** Science of the Total Environment 407 (2009) 5921–5925

Abstract

Investigations of environmental pollution by endocrine-disrupting chemicals are now in progress. Up to now, several *in vitro* bioassays have been developed for evaluation of the endocrine disruptive activity; however, there is still a lack of comparative studies of their sensitivity. In this work comparison of the oestrogen screening assay based on β-galactosidase expression and a bioluminescent oestrogen screen revealed differences in the sensitivity and specificity of the two tests. With the β-galactosidase screen a slight oestrogen-like activity of Delor 103, a commercial mixture of PCB congeners, and a fungicide triclosan was measured whereas no activity was detected using the bioluminescent assay. A bioluminescent androgen test negated previously suggested androgenic potential of triclosan.

Further, this work demonstrates the androgenic activity of Delor 103, with an EC50 value of 2.29×10−2mg/L. On the other hand, chlorobenzoic acids (CBAs), representing potential PCB degradation metabolites, exhibited no androgenic activity but were slightly oestrogenic. Their oestrogenicity varied with their chemical structure, with 2,3-CBA, 2,3,6-CBA, 2,4,6-CBA and monochlorinated compounds exhibiting the highest activity. Thus the results indicated possible transitions of the hormonal activity of PCBs during bacterial degradation.

Relevance assessment

Full text assessment of Svobodová K *et al*., 2009 has been conducted. The authors compared two *in vitro* assays - β-galactosidase assay (βgal test) and bioluminescent screens (lumino test) to study oestrogenic and androgenic activities of chlorobenzoic acids and chlorophenols. It was identified that chlorobenzoic acids exhibited no androgenic activity and slight oestrogenic activity. The applicant's considerations are as follows:

1. The methods were not validated. Deviation was large. Authors failed to show a negative control in figure comparing oestrogenic potential of chlorobenzoic acids.

2. Results were not consistent with other people's work or within this study; the two oestrogen assays came up with different results.

3. Some chlorobenzoic acids such as 2,3-CBA, 2,3,6-CBA, 2,4,6-CBA exhibited weak oestrogenic activity but other chlorobenzoic acids did not. However, the lack of a negative control makes the data difficult to interpret. Additionally, the authors claimed that the oestrogenicity varied with their chemical structure, but no structure-activity analysis was performed.

4. Ten chlorinated benzoic acids mentioned in the manuscript have been tested under the US EPA high-throughput EDSP program. The assessed compounds were inactive against the oestrogen, androgen, thyroid hormone and TSH receptors as well as against aromatase (CYP19A1), sodium/iodine symporter (NIS) and thyroid peroxidase (TPO) for all compounds with one exception for 4-chlorobenzoic acid with weak positive result (AC_{50} = 6.7 uM) obtained for the ACEA 80-hour impedance growth assay (using the T47D cell line).

However, this weak positive result is unlikely to represent a reliable oestrogenic response given the negative outcomes in other oestrogen receptor assays run with this compound that assess preceding key events in the AOP[19].

A summary table with the available EDSP data for the chlorinated benzoic acids was provided with this submission; the results for 4-chlorobenzoic acid are presented below:

[19] Browne P, Noyes PD, Casey WM, Dix DJ. Application of Adverse Outcome Pathways to U.S. EPA's Endocrine Disruptor Screening Program. Environ Health Perspect. 2017;125(9):096001. Published 2017 Sep 1. doi:10.1289/EHP1304

CAS	Name	Target / Receptor	Assay Name	Organism	Outcome
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	ACEA_ER_80hr	human	**Active**
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	NVS_NR_hER	human	Inactive
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	NVS_NR_mERa	mouse	Inactive
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	TOX21_ERa_BLA_Agonist_ratio	human	Inactive
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	TOX21_ERa_BLA_Antagonist_ratio	human	Inactive
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	TOX21_ERa_LUC_BG1_Agonist	human	Inactive
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	TOX21_ERa_LUC_BG1_Antagonist_Specificity	human	Inactive
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	TOX21_ERa_LUC_BG1_Antagonist	human	Inactive
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	TOX21_ERb_BLA_Antagonist_ratio	human	Inactive
74-11-3	4-Chlorobenzoic acid	Estrogen receptor (ER)	TOX21_ERb_BLA_Agonist_ratio	human	Inactive
74-11-3	4-Chlorobenzoic acid	Androgen receptor (AR)	NVS_NR_hAR	human	Inactive
74-11-3	4-Chlorobenzoic acid	Androgen receptor (AR)	NVS_NR_rAR	rat	Inactive
74-11-3	4-Chlorobenzoic acid	Androgen receptor (AR)	TOX21_AR_BLA_Agonist_ratio	human	Inactive
74-11-3	4-Chlorobenzoic acid	Androgen receptor (AR)	TOX21_AR_BLA_Antagonist_ratio	human	Inactive
74-11-3	4-Chlorobenzoic acid	Androgen receptor (AR)	TOX21_AR_LUC_MDAKB2_Agonist	human	Inactive
74-11-3	4-Chlorobenzoic acid	Androgen receptor (AR)	TOX21_AR_LUC_MDAKB2_Antagonist_Specificity	human	Inactive
74-11-3	4-Chlorobenzoic acid	Androgen receptor (AR)	TOX21_AR_LUC_MDAKB2_Antagonist	human	Inactive

CAS	Name	Target / Receptor	Assay Name	Organism	Outcome
74-11-3	4-Chlorobenzoic acid	Androgen receptor (AR)	TOX21_AR_LUC_MDAKB2_Agonist_Counterscreen	human	Inactive
74-11-3	4-Chlorobenzoic acid	Aromatase (CYP19A1)	TOX21_Aromatase_Inhibition	human	Inactive
74-11-3	4-Chlorobenzoic acid	Thyroid Hormone Receptor	TOX21_TR_LUC_GH3_Agonist	rat	Inactive
74-11-3	4-Chlorobenzoic acid	Thyroid Hormone Receptor	TOX21_TR_LUC_GH3_Antagonist	rat	Inactive
74-11-3	4-Chlorobenzoic acid	TSH Receptor	TOX21_TSHR_Agonist_ratio	human	Inactive
74-11-3	4-Chlorobenzoic acid	TSH Receptor	TOX21_TSHR_Antagonist_ratio	human	Inactive
74-11-3	4-Chlorobenzoic acid	TSH Receptor	TOX21_TSHR_wt_ratio	human	Inactive

Based on the considerations described above, it is concluded that there is no new information presented in this publication to inform data requirements, endpoints or risk assessments, thus the article is concluded to be non-relevant.

The study is not reliable; overall, the article would receive a Klimisch score of 3.

Score	Description	Details
3	Not reliable	"This includes studies or data from the literature/reports in which there are interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (e.g., unphysiological pathways of application) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for an assessment and which is not convincing for an expert judgment."

Conclusion

This review of the published literature for bixlozone and its metabolites did **not** reveal any studies considered to significantly affect the regulatory assessment of human health.

Nevertheless, attention is drawn by the applicant to the following:

- Metabolite 2 (2,4-Dichlorobenzoic acid, CAS number 50-84-0) is also a metabolite of the active substance spirodiclofen and is available commercially. It is also used as an intermediate for the manufacture of dyes, fungicides, pharmaceuticals and other chemicals.
- Metabolite 12 (3-hydroxypivalic acid, 4835-90-9) is also used as an intermediate in the manufacture of chemicals. A dossier to support the chemical under REACH was submitted in 2018 but does not appear to include any actual studies on toxicology or ecotoxicology.

References relied on

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
B.6.1.1.1/01 (KCA 5.1.1/01)	[redacted]	2014	Pharmacokinetics and metabolism of F9600 in male and female Sprague-Dawley rats [redacted] Report no. FMC-R2838 FMC Tracking no. 2013MET-ISX1020 Not to GLP Unpublished	Y	N	Not applicable	FMC	None (new active)
B.6.1.1.1/02 (KCA 5.1.1/02)	[redacted]	2017a	Metabolism of [^{14}C-phenyl]F9600 in male Sprague-Dawley rats - Pilot study [redacted] Study no. FMC-R3694 FMC Tracking no. 2014MET-ISX1323 Not to GLP Unpublished	Y	N	Not applicable	FMC	None (new active)
B.6.1.1.2/01 (KCA 5.1.1/04)	[redacted]	2016	Pharmacokinetics of [^{14}C-Phenyl]F9600 in Male and Female Sprague-Dawley Rats Following Single, Multiple Oral and Intravenous Bolus Doses [redacted], Study no. FMC-P3773 FMC Tracking no. 2014MET-ISX1628 GLP Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.1.1.4/01 (KCA 5.1.1/05)	[redacted]	2017b	Tissue distribution of [^{14}C-Phenyl]F9600 at peak concentration (T_{max}) in male and female Sprague-Dawley rats [redacted], Study no. FMC-P4973 FMC Tracking no. 2015MET-ISX2183 GLP Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.1.1.4/02 (KCA 5.1.3/02)	[redacted]	2017f	Radioactivity concentration in plasma and bone marrow at T_{max} after oral administration of [^{14}C]F9600 to Sprague-Dawley rats	Y	Y	Study to support new active approval in GB	FMC	None (new active)

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
			[redacted], Study no. FMC-P7354 FMC Tracking no. 2017MET-ISX3950 GLP Unpublished					
B.6.1.1.5/01 (KCA 5.1.1/06)	[redacted]	2018b	Excretion routes, mass balance and metabolism of [^{14}C-phenyl]F9600 in male and female Sprague-Dawley rats following single or multiple oral doses [redacted], Study no. FMC-P3887 FMC Tracking no. 2014MET-ISX1665 GLP Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.1.1.6/01 (KCA 5.1.1/03)	[redacted]	2018a	Metabolism of [^{14}C-carbonyl]F9600 in male and female Sprague-Dawley rats – pilot study [redacted] Report no. FMC-R3449 FMC Tracking no. 2014MET-ISX1303 Not to GLP Unpublished	Y	N	Not applicable	FMC	None (new active)
B.6.1.1.6/02 (KCA 5.1.1/07)	[redacted]	2017c	Excretion routes and metabolism of [^{14}C-carbonyl]F9600 in male and female Sprague-Dawley rats following a single oral dose [redacted] Study no. FMC-P4547 FMC Tracking no. 2015MET-ISX2296 GLP Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.1.3.1/01 (KCA 5.1.3/01)	[redacted]	2017e	Comparative *in vitro* Metabolism of [^{14}C] F9600 (Phenyl and Carbonyl-labelled) in mouse, rat, dog and human hepatocytes [redacted] Study no. FMC-R4547 FMC Tracking no. 2015MET-ISX2225 Not to GLP Unpublished	Y	N	Not applicable	FMC	None (new active)
B.6.1.3.2/01	[redacted]	2020	*In vitro* comparative metabolism [^{14}C-phenyl]-	Y	Y	Study to support new	FMC	None (new active)

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
(KCA 5.1.3/03)			and [^{14}C-carbonyl]-F9600 in mixed gender mouse, rat, dog, and human cryopreserved hepatocytes [redacted] Study no. FMC-P10690 FMC report no. FMC-53482 GLP Unpublished			active approval in GB		
B.6.1.3.2/02 (KCA 5.8.1/02)	Wijeysakere, S., Wang, W., Nallani, G., Guo, J., Jackson, S.A.	2020	Toxicological non-relevance of 4-hydroxymethyl F9600 (4-OH-Me-F9600) a disproportionate *in vitro* human metabolite of bixlozone FMC Corporation FMC report no. FMC-54077 Not to GLP, Unpublished	N	N	Not applicable	FMC	None (new active)
B.6.2.1/01 (KCA 5.2.1/01)	[redacted]	2014a	F9600 Technical: Acute Oral Toxicity – Up-and-Down Procedure in Rats [redacted] Study No.: 37755 FMC Tracking No.: 2013TOX-ISX0999 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.2.2/01 (KCA 5.2.2/01)	[redacted]	2014b	F9600 Technical: Acute Dermal Toxicity Study in Rats [redacted] Study No.: 37756 FMC Tracking No.: 2013TOX-ISX0998 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.2.3/01 (KCA 5.2.3/01)	[redacted]	2014c	F9600 Technical: Acute Inhalation Toxicity in Rats [redacted] Study No.: 37947 FMC Tracking No.: 2013TOX-ISX1000 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.2.4.1/01 (KCA 5.2.4/02)	Costin, G-E., Sheehan, D., Campasino, K.	2018	F9600 Technical: *In vitro* Skin Irritation Test (SIT) Using the Epiderm™ Skin Model Institute for *In Vitro* Sciences, Inc. Study No.: 18AC10.050082 FMC Tracking No.: 2018TOX-ISX4219 GLP, Unpublished	N	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.2.4.2/01	[redacted]	2014d	F9600 Technical: Primary Skin Irritation Study in	Y	Y	Study to support new	FMC	None (new active)

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
(KCA 5.2.4/01)			Rabbits [redacted] Study No.: 37758 FMC Tracking No.: 2013TOX-ISX0995 GLP, Unpublished			active approval in GB		
B.6.2.5.1/01 (KCA 5.2.5/02)	Wilt, N, Gamson, A.	2018	F9600 Technical: Epiocular™ Eye Irritation Test (EIT) for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage Institute for *In Vitro* Sciences, Inc. Study No.: 18AC10.015091 FMC Tracking No.: 2018TOX-ISX4220 GLP, Unpublished	N	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.2.5.2/01 (KCA 5.2.5/01)	[redacted]	2014e	F9600 Technical: Primary Eye Irritation in Rabbits [redacted] Study No.: 37757 FMC Tracking No.: 2013TOX-ISX0996 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.2.6/01 (KCA 5.2.6/01)	[redacted]	2014f	F9600 Technical: Local Lymph Node Assay (LLNA) in Mice [redacted] Study No.: 37759 FMC Tracking No.: 2013TOX-ISX0997 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.3.1.1/01 (KCA 5.8.2/02)	[redacted]	2015d	A 7-Day Oral (Dietary) Palatability Study of F9600 Technical in Sprague Dawley Rats [redacted] Study No.: [redacted]-105106 FMC Tracking No.: 2013TOX-ISX1063 Not to GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.3.1.2/01 (KCA 5.8.2/01)	[redacted]	2015c	A 7-Day Oral (Dietary) Palatability Study of F9600 in CD-1 Mice [redacted] Study No.: [redacted]-105107 FMC Tracking No.: 2013TOX-ISX1064 Not to GLP, Unpublished	Y	N	Not applicable	FMC	None (new active)
B.6.3.1.3/01	[redacted]	2015e	A 7-Day Oral (Dietary) Palatability Study of F9600	Y	N	Not applicable	FMC	None (new active)

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
(KCA 5.8.2/03)			Technical in Beagle Dogs [redacted], Study No.: [redacted]-105126 FMC Tracking No.: 2014TOX-ISX1192 Not to GLP, Unpublished					
B.6.3.1.4/01 (KCA 5.3.1/04)	[redacted]	2016b	A 7-Day Oral (Capsule) Toxicity Study of F9600 Technical in Beagle Dogs. [redacted] & [redacted] Study no. [redacted]-105154 FMC Tracking no. 2014TOX-ISX1623 Not to GLP Unpublished	Y	N	Not applicable	FMC	None (new active)
B.6.3.2.1/01 (KCA 5.3.1/01)	[redacted]	2015a	A 28-Day Oral (Dietary) Toxicity and Toxicokinetic Study of F9600 Technical in Sprague Dawley Rats [redacted] & [redacted] Study No.: [redacted]-105108 FMC Tracking No.: 2013TOX-ISX1073 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.3.2.2/01 (KCA 5.3.1/02)	[redacted]	2015b	A 28-Day Oral (Dietary) Toxicity Study of F9600 Technical in CD-1 Mice [redacted] & [redacted] Study No.: [redacted]-105109 FMC Tracking No.: 2013TOX-ISX1074 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.3.2.3/01 (KCA 5.3.1/03)	[redacted]	2016a	A 28-Day Oral (Dietary) Toxicity Study of F9600 Technical in Beagle Dogs [redacted] & [redacted] Study No.: [redacted]-105117 FMC Tracking No.: 2013TOX-ISX1087 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.3.3.1/01 (KCA 5.3.2/01)	[redacted]	2016a	A 90-Day Dietary Combined Toxicity and Neurotoxicity Study of F9600 in Rats [redacted]	Y	Y	Study to support new active approval in GB	FMC	None (new active)

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
			Study No.: [redacted]-105119 FMC Tracking No.: 2013TOX-ISX1085 Amdt 1 GLP, Unpublished					
B.6.3.3.2/01 (KCA 5.3.2/02)	[redacted]	2016b	A 90-Day Oral (Dietary) Toxicity and Plasma Concentration Measurement Study of F9600 Technical in CD-1 Mice [redacted] Study No.: [redacted]-105118 FMC Tracking No.: 2013TOX-ISX1086 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.3.3.3/01 (KCA 5.3.2/03)	[redacted]	2016c	A 90-Day Oral (Capsule) Dose Toxicity Study of F9600 Technical in Beagle Dogs [redacted] Study No. [redacted]-105124 FMC Tracking No.: 2013TOX-ISX1088 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.3.3.3/02 (KCA 5.8.3/02)	Anonymous	2020	Complied - Rat Rabbit Mouse Dog HCD – Bixlozone Charles River Laboratories Ashland, LLC FMC report no. FMC-54569 Not to GLP, Unpublished	N	N	Not applicable	FMC	None (new active)
B.6.3.4/01 (KCA 5.3.2/04)	[redacted]	2017	A 12-Month Oral (Capsule) Dose Toxicity Study of F9600 Technical in Beagle Dogs [redacted] Study no. [redacted]-105125 FMC Tracking no. 2013TOX-ISX1091 GLP Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.3.5/01 (KCA 5.3.3/01)	[redacted]	2016	A 21-Day study of F9600 by Dermal Application in Sprague-Dawley Rats [redacted] Study no. [redacted]-105181 FMC Tracking no. 2016TOX-ISX2425 GLP Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.4.1.1/01 (KCA 5.4.1/01)	Bruce, S	2018	Bacterial Reverse Mutation Assay with F9600 Technical BioReliance Corporation,	N	Y	Study to support new active approval in	FMC	None (new active)

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
			Rockville, MD, Study no. BioReliance AE80XH.503.BTL FMC Tracking no. 2016TOX-ISX2999 GLP Unpublished			GB		
B.6.4.1.2/01 (KCA 5.4.1/02)	Roy, S	2018	*In Vitro* Mammalian Chromosomal Aberration Assay in Chinese Hamster Ovary (CHO) Cells with F9600 Technical BioReliance Corporation, Rockville, MD, Study no. BioReliance AE80XH.331.BTL FMC Tracking no. 2016TOX-ISX3672 GLP Unpublished	N	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.4.1.3/01 (KCA 5.4.1/03)	Dutta, A	2018	*In Vitro* Mammalian Cell Gene Mutation Test (L5178Y/TK+/- Mouse Lymphoma Assay) with F9600 Technical BioReliance Corporation, Rockville, MD, Study no. BioReliance AE80XH.704.BTL FMC Tracking no. 2017TOX-ISX3992 GLP Unpublished	N	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.4.2/01 (KCA 5.4.2/01)	[redacted]	2018	*In Vivo* Mammalian Erythrocyte Micronucleus Assay in Rats with F9600 Technical [redacted] Study no. [redacted] AE80XH.125M021.BTL FMC Tracking no. 2017TOX-ISX3267 GLP Unpublished	N	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.5.1/01 (KCA 5.5/01)	[redacted]	2017	A 2-Year Oral (Dietary) Combined Chronic Toxicity and Carcinogenicity Study with Toxicokinetic Measurements of F9600 Technical in Sprague Dawley Rats. [redacted]	Y	Y	Study to support new active approval in GB	FMC	None (new active)

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
			[redacted]. Study no. [redacted]-105121 FMC Tracking no. 2013TOX-ISX1089 GLP Unpublished					
B.6.5.1/02 (KCA 5.5/03)	Thomas, H.C.	2020	Pathology working group review of selected neoplasms from rats in an 2-year oral (dietary) combined chronic toxicity and carcinogenicity study with toxicokinetic measurements of F9600 technical Experimental Pathology Laboratories, Inc. Study no. 115-021 FMC report no. FMC-53830 Not to GLP Unpublished	N	N	Not applicable	FMC	None (new active)
B.6.5.2/01 (KCA 5.5/02)	[redacted]	2017	An 18-month Oral (Dietary) Carcinogenicity Study with Toxicokinetic Measurements of F9600 Technical in CD-1 Mice [redacted] Study no. [redacted]-105120 FMC Tracking no. 2013TOX-ISX1090 GLP Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.5.2/02 (KCA 5.5/04)	Thomas, H.C.	2020	Pathology working group review of selected neoplasms from mice in an 18-month oral (dietary) carcinogenicity study with toxicokinetic measurements of F9600 technical Experimental Pathology Laboratories, Inc. Study no. 115-020 FMC report no. FMC-53526 Not to GLP Unpublished	N	N	Not applicable	FMC	None (new active)
B.6.5.2/03 (KCA 5.5/05)	Thomas, H.C., Patrick, D.J.	2020	Bixlozone: Response to the UK HSE Assessment of tumors of the cervix in the mouse 18-Month oral carcinogenicity study Experimental Pathology Laboratories, Inc. FMC report no. FMC-55283	N	N	Not applicable	FMC	None (new active)

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
			Not to GLP Unpublished					
B.6.6.1.1/01 (KCA 5.6.1/01)	[redacted]	2016b	A Dose Range Finding Oral (Dietary) Reproduction/Developmental Study of F9600 Technical in Sprague-Dawley Rats [redacted] Study No.: [redacted]-105133 FMC Tracking No.: 2014TOX-ISX1294 Not to GLP, Unpublished	Y	N	Not applicable	FMC	None (new active)
B.6.6.1.2/01 (KCA 5.6.1/02)	[redacted]	2016c	A Dietary Two-Generation Reproductive Toxicity Study of F9600 Technical in Rats [redacted] Study No.: [redacted]-105134 FMC Tracking No.: 2014TOX-ISX1295 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.6.2.1/01 (KCA 5.6.2/01)	[redacted]	2016d	An Oral (Gavage) Dose Range-Finding Prenatal Developmental Toxicity Study of F9600 in Rats [redacted] Study No.: [redacted]-105129 FMC Tracking No.: 2014TOX-ISX1290 Not to GLP, Unpublished	Y	N	Not applicable	FMC	None (new active)
B.6.6.2.2/01 (KCA 5.6.2/02)	[redacted]	2016e	An Oral (Gavage) Prenatal Developmental Toxicity Study of F9600 in Rats [redacted] Study No.L [redacted]-105130 FMC Tracking No.: 2014TOX-ISX1291 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.6.2.3/01 (KCA 5.6.2/03)	[redacted]	2014a	An Oral (Gavage) Dose Range-Finding Prenatal Developmental Toxicity Study of F9600 Technical in Rabbits [redacted] Study No.: [redacted]-105131 FMC Tracking No.:	Y	N	Not applicable	FMC	None (new active)

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
			2014TOX-ISX1292 Not to GLP, Unpublished					
B.6.6.2.4/01 (KCA 5.6.2/04)	[redacted]	2015	An Oral (Gavage) Prenatal Developmental Toxicity Study of F9600 Technical in Rabbits [redacted] Study No.: [redacted]-105132 FMC Tracking No.: 2014TOX-ISX1293 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.7.1/01 (KCA 5.7.1/01)	[redacted]	2014a	An Oral (Gavage) Dose Range Finding Acute Neurotoxicity Study of F9600 Technical in Rats [redacted] Study No.: [redacted]-105113 FMC Tracking No.: 2013TOX-ISX1065 Not to GLP, Unpublished	Y	N	Not applicable	FMC	None (new active)
B.6.7.2/01 (KCA 5.7.1/02)	[redacted]	2014b	An Oral (Gavage) Acute Neurotoxicity Study of F9600 Technical in Rats [redacted] Study No.: [redacted]-105114 FMC Tracking No.: 2013TOX-ISX1066 GLP, Unpublished	Y	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.8.1.1/01 (KCA 5.8.1/09)	Gilby, B.	2021a	2,4-Dichlorobenzoic Acid: Bacterial Reverse Mutation Test Labcorp Early Development Laboratories Ltd. U.K., Report no. FMC-55761 GLP, Unpublished	N	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.8.1.1/02 (KCA 5.8.1/07)	Gilby, B.	2021b	2,4-Dichlorobenzoic Acid: In Vitro Mammalian Cell Micronucleus Test in Human Peripheral Lymphocytes Covance Laboratories Limited U.K., Report no. FMC-55762 GLP, Unpublished	N	Y	Study to support new active approval in GB	FMC	None (new active)
B.6.8.1.1/03 (KCA 5.8.1/08)	Gilby, B.	2021c	2,4-Dichlorobenzoic Acid: In Vitro Hprt Mutation Test Using Chinese Hamster Ovary Cells Labcorp Early Development Laboratories Ltd. U.K., Report no. FMC-55811 GLP, Unpublished	N	Y	Study to support new active approval in GB	FMC	None (new active)

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B.6.8.1/01 (KCA 5.8.1/01)	Jackson, S.A.	2019	Assessment and in-silico Toxicology Predictions for Metabolites of Bixlozone FMC Corporation FMC Tracking No.: 2018WHP-ISX4394 Not to GLP, Unpublished	N	N	Not applicable	FMC	None (new active)
B.6.8.1/02 (KCA 5.8.1/03)	Wijeyesakere, S.J., Wang, W., Guo, J., Nallani, G., Jackson, S.A.	2020	Assessment of the toxicological relevance of metabolites of bixlozone (F9600) Undertaken in support of active substance review in Europe FMC Corporation FMC report no. FMC-55114 Not to GLP, Unpublished	N	N	Not applicable	FMC	None (new active)
B.6.8.1/03 (KCA 5.8.1/04)	Benigni, R., Bossa, C.	2011	Mechanisms of chemical carcinogenicity and mutagenicity: a review with implications for predictive toxicology Chem Rev. 111(4):2507-2536 Not to GLP, Published	N	N	Not applicable	Authors	None (new active)
B.6.8.1/04 (KCA 5.8.1/06)	Cramer, G.M., Ford, R.A., Hall, R.L.	1976	Estimation of toxic hazard-A decision tree approach Food Cosmet. Toxicol., 16 (3), 255–276 Not to GLP, Published	N	N	Not applicable	Authors	None (new active)
B.6.8.4/01 (KCA 5.8.3/01)	Wholman, I.; Kung, T.; Clerkin, D	2020	F9600: Assessment of Experimental Data to Characterize Evidence of Endocrine Disrupting Potential FMC Tracking No.: 2018WHP-ISX4276, Revision No. 2 and FMC-52696, Revision No. 1 Not to GLP, Unpublished	N	N	Not applicable	FMC	None (new active)
B.6.10/01 (KCA 5.8.3/07)	Svobodová K., Plačková M., Novotná V., Cajthaml T.	2009	Oestrogenic and androgenic activity of PCBs, their chlorinated metabolites and other endocrine disruptors estimated with two *in vitro* yeast assays Science of the Total Environment 407 (2009) 5921–5925 Not to GLP, Published	N	N	Not applicable	Authors	None (new active)