Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

EC Number: **255-894-7**

CAS Number: **42576-02-3**

Index Number: **-**

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Polish Competent Authority,

Bureau for Chemical Substances

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CONTENTS

SUPPORT ON HOW TO COMPILE ANNEX I TO THE CLH REPORT

Annex I to the CLH report may be compiled from DARs, CARs and/or other sources. Non-confidential DAR/CAR can be annexed as such provided that it has sufficient level of details on the studies. The DS is encouraged to remove any irrelevant parts of the DAR/CAR. The DS must ensure that Annex I can be published during PC, i.e. it does not contain any confidential information.

For support, below is an example on how each study could be presented individually under its own subchapter including the study reference, detailed study summary and results. The format of the detailed study summary of an individual study is flexible as long as the summary is clearly reported and under a correct hazard class. Detailed support can be found below under each subchapter. If DAR/CAR is annexed to the CLH report as Annex I, it must be indicated clearly in the evaluation part of the report where in Annex I the relevant study can be found. If read-across to structurally or mechanistically similar substance is used please provide a justification for using data from this substance and, if known, present the calculations to convert dose/concentration levels from the test substance to the substance for which CLH is proposed. Please provide also a justification for providing non-testing data by any other approaches such as quantitative structure-activity relationships (QSARs) or grouping methods. Support on grouping of substances and read-across can be found in the following links:

http://echa.europa.eu/documents/10162/13632/information_requirements_r6_en.pdf

http://echa.europa.eu/documents/10162/13655/pg_report_qsars_en.pdf

http://echa.europa.eu/documents/10162/13655/pg_report_readacross_en.pdf

http://www.qsartoolbox.org/

http://www.oecd.org/chemicalsafety/risk-assessment/groupingofchemicalschemicalcategoriesandread-across.htm

http://echa.europa.eu/en/view-article/-/journal_content/title/assessing-read-across-how-echa-does-it

1 PHYSICAL HAZARDS

1.1 Explosives

1.1.1 Study 1 - CA 2.11 Explosive properties

Study 1 reference

Anonymous; Determination of the Explosion Properties, Ability for Self-Heating and Oxidizing Properties of Technical Bifenox; 1998

Test type

EEC Directive 92/69 Appendix V Part A.14
The corresponding study was performed under GLP

Detailed study summary and results:

Test material: Bifenox technical (lot no:. OP9750096)
The determination of explosion properties of technical Bifenox was performed using thermal sensitivity test apparatus, shock test and friction test apparatus in accordance with EEC Directive 92/69 Appendix V part A14 except that the thermal sensitivity test was conducted with the modification to the tube packing procedure as detailed in "Safety characteristics data of explosive substances – Methods of the Bundesanstalt für material prüfung –BAM" KOENEN, IDE and SWART.
It has been determined that Bifenox technical does not have shock sensibility to explosion, does not have friction sensibility to explosion and does not have thermal sensibility to explosion.
Based on the determined test, it can be concluded that the test item Bifenox technical does not present a danger of explosion under the conditions of the test.

Material and methods

The determination of explosive properties was conducted using following systems:
- The BAM Fall Hammer (or shock) for measurement of mechanical sensitivity (shock)
- The BAM Friction apparatus for measurement of mechanical sensitivity (friction)
- The steel tube apparatus for measurement of thermal sensibility

Results

- *numerical results (mean value and repeatability) for all tests and controls:*
- *thermal sensitivity:*
  The results of all measurements were: No explosion
- *mechanical sensitivity*
  The results of all measurements were: No ignition, no explosion
- *sensitivity to friction*
  The results of all measurements were: No ignition, no explosion
- *explosive or non explosive*
  The test item is considered as non explosive

1.2 Flammable gases (including chemically unstable gases)

Data waiving

Study technically not feasible.

Justification for data waiving

The test item is not a gas. The determination of flammability of gases is technically not feasible to be conducted.

1.3 Oxidising gases

Data waiving

Study technically not feasible.

Justification for data waiving

The test item is not a gas. The determination of oxidising properties of gases is technically not feasible to be conducted.

1.4 Gases under pressure

Data waiving

Study technically not feasible.

Justification for data waiving

The test item is not a gas. The conduction of the corresponding study is technically not feasible.

1.5 Flammable liquid

Data waiving

Study technically not feasible.

Justification for data waiving

The test item is a solid. The conduction of the corresponding study is technically not feasible.

1.6 Flammable solids

1.6.1 Study 1 - CA 2.9 Flammability and self-heating

Study 1 reference:

Anonymous; Bifenox – Determination of the Flammability; 2000

Test type

EEC Directive 92/69 Appendix V Part A.10
The corresponding study was performed under GLP

Detailed study summary and results:

Test item: Bifenox (lot no:. OP9850224)
The determination of the flammability of Bifenox was performed using train a 10 mm long, by 250 mm width by 10 mm high in accordance with EEC Directive 92/69 Appendix V Part A,10 except that the ignition source was a hot platinum wire (≥ 1000 as proposed in NF T 20-042 (Sep. B5). Bifenox is not flammable under the conditions of the test.

Material and methods

- *Indicate if preliminary and/or main test performed*
  No information is provided
- *Moisture content*
  No information is provided
- *Particle size and distribution*
  No information is provided

Results

The test item melts when in contact when in contact with hot wire, but no flame is observed No further testing was required. Bifenox is not highly under the conditions of the test.

1.6.2 Study 2 - CA 2.9 Flammability and self-heating

Study 2 reference:

Anonymous; Determination of the Explosion Properties, Ability for Self-Heating and Oxidizing Properties of Technical Bifenox; 1998

Test type

EEC Directive 92/69 Appendix V Part A.16
The corresponding study was performed under GLP

Detailed study summary and results:

Test material: Bifenox technical (lot no:. OP9750096)
The determination of the relative self-ignition temperature of technical Bifenox was performed in accordance with EEC Directive 92/69 Appendix V part A.16. The test item Bifenox technical melted at about 89°C. No auto ignition occurred. A melting point was reached, no further investigation was required.

Material and methods

- *Indicate if preliminary and/or main test performed*
  No detailed information is provided
- *Moisture content*
  No information for the corresponding test provided
- *Particle size and distribution*
  No information for the corresponding test provided

Results

The temperature of the oven at which the sample substance reaches 400 °C by heating is relevant for evaluation of the substance does not melt before. In the corresponding study technical Bifenox melted at about 89 °C. No auto ignition occurred so that no further investigation was required.

1.7 Self-reactive substances

Data waiving

Study scientifically not necessary / other information available.

Justification for data waiving

The study does not need to be conducted because there are no chemical groups present in the molecule which are associated with the explosive of self-reactive properties and hence the classification procedure does not need to be applied.

1.8 Pyrophoric liquids

Data waiving

Study technically not feasible.

Justification for data waiving

The test item is solid. The conduction of the corresponding study is technically not feasible.

1.9 Pyrophoric solid

Data waiving

Study scientifically not necessary / other information available.

Justification for data waiving

The study does not need to be conducted because the substance is known to be stable in contact with air at room temperature for prolonged periods of time (days), hence the classification procedure does not need to be applied.

1.10 Self-heating substances

Data waiving

Study scientifically not necessary / other information available.

Justification for data waiving

The study does not need to be conducted because the substance is known to be stable in contact with air at room temperature for prolonged periods of time (days). Furthermore, the melting point of the test item is 86.0 to 87.7 ºC (< 160 °C), what drastically reduce the substance air surface (endothermic process) and excludes the consideration of the substance as a self-heating substance. Hence, the classification procedure does not need to be applied.

1.11 Substances which in contact with water emit flammable gases

Data waiving

Study scientifically not necessary / other information available.

Justification for data waiving

The study does not need to conducted because the organic substance does not contain metals or metalloids and hence, the classification procedure does not need to be applied.

1.12 Oxidising liquids

Data waiving

Study technically not feasible.

Justification for data waiving

The test item is a solid. The conduction of the corresponding study is technically not feasible.

1.13 Oxidising solids

1.13.1 Study 1 - CA 2.13 Oxidising properties

Study 1 reference:

Anonymous; Determination of the Explosion Properties, Ability for Self-Heating and Oxidizing Properties of Technical Bifenox; 1998

Test type

EEC Directive 92/69 Appendix V Part A.17
The corresponding study was performed under GLP

Detailed study summary and results:

Test material: Bifenox technical (lot no:. OP9750096)
The determination of the oxidising properties of technical Bifenox was performed using a powder train about 250 mm long, by 20 mm wide, by 10 mm high. Barium nitrate is used as a reference substance. The sample is mixed with a combustible substance, being powdered cellulose. This is in accordance with EEC Directive 92/69 Apppendix V part A.17 except that the ignition source was a hot wire (≥ 1000°C) as proposed in NF T 20-035. The test item was found not to have any oxidising properties.

Material and methods

- *Particle size and distribution*
  The test substance is reduced to a particle size (< 125 µm) by sieving through an analytical sieve, and dried in an over at 105°C until constant weight.
- *Test material identity*
  Bifenox technical (lot no:. OP9750096)
- *Sample preparation (e.g. grinding, sieving, drying)*
  Test substance / cellulose mixtures containing 10 – 90% test substance in 10% increments weight are prepared. As a reference, a barium nitrate / cellulose mixture containing 2/1 (w/w) barium nitrate is also prepared
- *Reference substance*
  Barium nitrate is blended and reduced to a particle size (< 125 µm)
- *Combustible substance and drying procedure used*
  Powdered cellulose is prepared in the same way as the test substance
- *Preliminary and/or main test used*

Both tests were performed

Results

Preliminary test:

Concerning the reference mixture (substance / cellulose – ratio 2/1) no flame propagation is observed)

Main study:

The combustion speed of cellulose 40% / barium bitrate 60% is 0.58 mm/s.
90/10 and 80/20 cellulose/substance amounts: limited flame propagation after ignition.
70/30, 60/40 and 50/50 cellulose / substance amounts: combustion time is lower than that of cellulose / barium nitrate test.
Other amounts: the product melts, but there is no flame propagation.
The maximum combustion speed of cellulose / substance amounts is 0.30 mm/s (50/50 cellulose/substance amounts), therefore the test item is considered as having no oxidising properties.

1.13.2 Study 2 - CA 2.13 Oxidising properties

Study 2 reference:

Anonymous; Bifenox Technical, Oxidising properties A.17; 2006

Test type

Council Directive 67/548/EEC Annex V, Part A: Methods for the determination of physico-chemical properties, A.17 Oxidising properties
The corresponding study was performed under GLP

Detailed study summary and results:

Test material: Bifenox technical (batch no:. 040724, purity 97.8%)

The determination of the oxidising properties of technical Bifenox was performed using a metal mould having a length of 250 mm and a triangular cross-section with an inner height of 10 mm and an inner width of 20 mm. First test was performed with the reference substance barium nitrate. The test was then carried out at least once with each one of the range of mixture of the test item with cellulose. Additional experiments in an inert atmosphere (Argon) and with inert substance (Kieselguhr) were performed. For the experiments in the inert Atmosphere (Argon) a hot platinum wire was used as ignition source.
This test is performed according to Council Directive 67/548/EEC Annex V, Part A: Methods for the determination of physic-chemical properties, A.17 Oxidising properties. The test item was found not to have oxidising properties.

Material and methods

- *Particle size and distribution*
  The test item is sieved < 0.125 mm
- *Test material identity*
  Bifenox technical (lot no:. 040724, purity 97.8%)
- *Sample preparation (e.g. grinding, sieving, drying)*
  The cellulose was dried by treating the sample at 105 ± 5°C until the weight was constant. Due to the particle size of the cellulose grinding and sieving was not necessary. The barium nitrate was ground, sieved < 0.125 mm and dried by treating the sample at 105 ± 5°C until the weight was constant. The kieselguhr was dried by treating the sample at 105 ± 5°C until the weight was constant. Due to the particle size of the kieselguhr grinding and sieving was not necessary. The test item was ground, sieved < 0.125 mm and dried by treating the sample at 50 ± 5°C under vacuum (10^{-3} hPa) until the weight

was constant. The drying temperature was chosen due to the melting of the test item starting at approx. 84°C.

- *Reference substance*
  Barium nitrate (supplier Merck KGaA, Darmstadt)
  Reagents:
  Cellulose microcrystalline (Avicel®) (supplier Merck KGaA, Darmstadt)
  Kieselguhr (Riedel-de Haen)
- *Combustible substance and drying procedure used*
  See above
- *Preliminary and/or main test used*
  Both tests were performed

Results

Preliminary test:

Two tests mixture of the test item and cellulose 2:1 burned down in 245 and 195s. Three tests mixture of barium nitrate and cellulose 2:1 burned down in 93, 90 and 87 s. Since the mixture of test item and cellulose burned slower compared to the mixture of barium nitrate and cellulose the full test was necessary.

Main study:

Five tests were performed for the reference mixture (Barium nitrate and Cellulose) with 40, 50, 55, 60 and 70 wt. % oxidizer (barium nitrate). The highest burning rate 1.14 mm/s was found for the mixture with 55 wt. % oxidiser.
Mixtures of the test item with cellulose were prepared in 10% increments between 10 and 60 wt. % test item and tested. Additional tests with 15, 25, 80 and 100 wt.% test item were performed. The highest burning rate 1.24 mm/s was found for the mixture with 25% test item. Due to the fact that the highest burning rate of the test item and cellulose (1.24 mm/s) was higher than the highest burning rate of the mixture with reference substance and cellulose (1.14 mm/s) the test was stopped and no further tests with the three mixtures having shown the highest burning rate were performed.
To exclude a false positive result, additional experiments of the test item and of barium nitrate (55 wt. %) in mixture with kieselguhr were performed.
For the pure test item the test item melted when a flame was applied as ignition source. The melt did not burn due to the limited surface of the melt.
The mixtures of 50 -70 wt. % of the test item with kieselguhr showed a short local combustion of the test item. Due to the fact that kieselguhr is an inert substance and does not burn it has to be concluded from this test, that the melted test item sustained the combustion if soaked in a porous material. In mixture with kieselguhr the melted test item was soaked in the porous kieselguhr and let to an increase of the free surface, which sustained the combustion. From these tests it can be concluded that also in mixture with cellulose the melted test item was soaked in the porous structure of cellulose and sustained the combustion.
Additional tests in an inert atmosphere (Argon) with a glowing platinum wire as ignition source were performed in order to prevent a false positive result. A mixture of 25 wt. % of test item with cellulose showed that the mixture of the test and cellulose could be ignited with a glowing platinum wire. The reference mixture burned down completely in 247 s (0.81 mm/s). Due to the burning behaviours of the mixture of test item kieselguhr and in an inert atmosphere, it is concluded that the test item has no oxidising properties.

1.14 Organic peroxides

Data waiving

Study scientifically not necessary / other information available.

Justification for data waiving

The study does not need to be conducted because the test item does not fall under the definition of organic peroxides according to GHS and the relevant UN Manual of tests and criteria.

1.15 Corrosive to metals

Data waiving

Study scientifically not necessary / other information available.

Justification for data waiving

The study does not need to be conducted because the test item does not contain chemical groups, which could initiate an irreversible electrochemical reaction with metals leading to significant damage or destruction.

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

2.1.1 Study 1 - CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure - Comparative *in vitro* metabolism

Report:	KCA 5.1.1/01 Anonymous (2015)
Title:	Comparative *in vitro* metabolism of [^{14}C]-Bifenox using rat and human liver microsomes
Document No:	TNO Triskelion, 20664
Guidelines:	Not applicable
GLP	Yes

Detailed study summary and results:

Test substance and experimental procedure

The study was designed to compare the *in vitro* metabolism of ring labelled [^{14}C]-Bifenox (batch # CFQ42207) by liver microsomes of Wistar rats and humans. Metabolic activity of the microsomes towards a standard substrate (testosterone) was determined and found appropriate.

The average recovery of radioactivity was 99.1%, of which 97.2% was present in the supernatant and 1.8% in the pellet. The final incubations with rat and human liver microsomes were conducted for 10, 20 and 30 minutes at 37ºC, with 10 µM [^{14}C]-Bifenox and 0.1 mg/mL microsomal protein. The reactions were terminated by the addition of acetonitrile. [^{14}C]-Bifenox and metabolite peaks were detected using HPLC with on-line radioactivity detection.

In preliminary experiments, it was demonstrated that in buffer solution without microsomes, [^{14}C]-Bifenox was stable. Addition of microsomes showed a high rate of degradation of Bifenox into Bifenox acid, the hydrolysis product of Bifenox. Addition of NADPH, which represents biological metabolism, showed no metabolite formation in addition to Bifenox acid.

Results

Metabolic activity of the microsomes towards a standard substrate (testosterone) was determined and found appropriate. The homogeneity of the dose solutions was checked. The results show that the dose solutions were homogeneous. The average [^{14}C]-Bifenox concentration in the dose solutions was 1.03 mM, giving an incubation concentration of 10.3 µM. The average recovery of radioactivity in the recovery experiment was 99.1%, of which 97.2% was present in the supernatant and 1.8% in the pellet.

In preliminary experiments, it was demonstrated that rat and human liver microsomes show similar patterns. [^{14}C]-Bifenox (eluting at 27.6 min) degrades very quickly and complete in buffer into a metabolite eluting at 24.0 min. Addition of

NADPH had no effect on the metabolite pattern. Almost complete degradation occurred with 0.2 and 1.0 mg/mL microsomal protein. Additional incubations were performed with 0.01, 0.04 and 0.1 mg/mL microsomal protein. Based on these results, it was decided to incubate [^{14}C]-Bifenox at 10 µM for 10, 20 and 30 minutes using a microsomal protein concentration of 0.01 mg/mL. Since addition of NADPH had no effect on the metabolite pattern, incubations in the main study were performed without NADPH.

Human microsomal extracts were spiked with the reference metabolites Amino-Bifenox, Amino-Bifenox acid and Bifenox acid. It was clearly confirmed, that the metabolite formed was Bifenox acid, the hydrolysis product of Bifenox. The stability of Bifenox in buffer for 30 minutes was demonstrated.

The addition of microsomes at 0.01 mg/mL microsomal protein showed high percentage (70%) of Bifenox acid at 10 minutes. Results for rat and human liver microsomes were similar. After 10 minutes, Bifenox acid formation seemed to be complete, although the percentage of Bifenox acid in rat liver microsomes slightly increased to 87% at 30 minutes.

Table 2.1.1-1 [^{14}C]-Bifenox metabolite formation in rat and human liver microsomes

Time	NADPH 3 mM	MICs 0.01 mg/mL	% Peak area			
			RT 24.0 min (Bifenox acid)		RT 27.6 min (Bifenox)	
			Rat	Human	Rat	Human
30 min	-	-	-	-	100	100
10 min	-	+	69.4	71.5	30.6	28.5
20 min	-	+	69.9	75.7	30.1	24.3
30 min	-	+	86.6	73.5	13.4	26.5

Time Incubation time 10 µM [^{14}C]-Bifenox
MICs microsomes
RT retention time (HPLC)
- not detected

The observed transformation of [^{14}C]-Bifenox in the test system was due to protein reactivity and/or ester hydrolysing enzymes. NADPH dependent metabolism of [^{14}C]-Bifenox and its metabolite Bifenox acid could not be observed under the conditions applied. Reactions in rat and human liver microsomes were similar and no unique human metabolite was observed.

2.1.2 Study 2 - CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure

Report:	Anonymous (1986)
Title:	Biokinetics and metabolism in the male and female rat
Document No:	26/08/25
Guidelines:	The study is in accordance with EEC 87/302 Annex V B or OECD 417 (1984)
GLP	Yes

Detailed study summary and results:

Test type

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool) has been used for evaluation. This study is categorized into Klimisch[1] category 1: reliable without restriction. Therefore, it is concluded that this study is valid, and is considered for classification.

In order to confirm relevant information on adsorption, distribution, metabolism and excretion of Bifenox, this study comprised a series of experiments using male and female rats that were administered an oral dose of radio-labelled

[1] Klimisch et al., 1997, Regulatory Toxicology Pharmacology 25(1), 1-5

Bifenox. Low and high dose, single and repeated dosing was investigated, following evaluation of Bifenox concentrations from samples or tissues involved in ADME.

Test substance and experimental procedure

Bifenox dichloro-ring uniformly [^{14}C]-labelled (batch #. ICN 989292, radiochemical purity <96%) were diluted with non-radiolabelled Bifenox (batch # SRI-167-82-3, purity 99.8%) to a final specific activity of 0.25 mCi/mmole. The active substance was dissolved in aqueous gum tragacanth (0.25% w/v). Fresh dose suspensions were prepared on days 0, 6 and 10. The stability of the dose suspension over the 6-day period was assessed prior to the study.

Five Charles River CD rats/sex/dose were dosed by gavage at single dose of 90 or 900 mg/kg. 8 rats/sex received repeated dosing of 14 × 90 mg/kg bw and on day 15 with radiolabelled material. Animals were fasted before dosing. Urine and faeces were collected; exhaled air traps were emptied at 4, 8, 24 and 48 h. Blood samples were taken prior to dosing and at 0.25, 0.5, 1, 2, 3, 5, 7, and 24 h and at 24 h intervals until 168 h.

Results

Absorption

The recovery of administrated radioactivity was about 100% in the high dose groups within seven days. Slightly lower value was found for males of the low dose group whereas radioactivity recovered from females was about 100%. Comparison of the excreted radioactivity following the single oral low and high doses revealed no significant difference. Absorption was not affected by repeated doses as was evidenced by a recovery of administrated radioactivity of 98.55% (males) and 96.56% (females), respectively.

Table 2.1.2-1 Recovery of [^{14}C]-Bifenox in the rat

Sex	Treatment	Dose [mg/kg]	Urine	Faeces	Total recovery
Males	Single oral	90	29.11%	63.41%	92.52%
Females	Single oral	90	52.64%	46.24%	98.88%
Males	Single oral	900	11.67%	84.90%	96.57%
Females	Single oral	900	19.56%	82.46%	102.02%
Males	Repeated oral	90	20.50%	78.05%	98.55%
Females	Repeated oral	90	38.52%	58.04%	96.56%

Distribution

Seven days after dosing only low concentrations were found in blood or tissue, mainly in the liver and the kidney. Mean values for radioactivity in the kidney were 0.62 ppm for males and 0.46 for females after repeated oral administration of 90 mg/kg. Similar levels were found in the liver. Thus, there is no evidence of retention in any tissue or organ. No significant difference of residual radioactivity values were observed after single or repeated dosing at 90 mg/kg. However, slightly increased values were observed in animals of the high dose group. There were also no significant difference of residual radioactivity for males and females.

Excretion

Excretion via urine was more pronounced after single and repeated oral treatment with low doses. Single oral high dose regime changes the ratio urine/faeces excretion in favour of the latter. Females excreted a greater portion of radioactivity administered in the urine than did males.

Metabolism

Radioactivity detected in urine for all groups and both sexes was associated mainly with 5-(2,4-dichlorophenoxy)-2-nitrobenzoic acid. No Bifenox was detectable in urine. In faeces excreted radioactivity was associated mainly with Bifenox and 5-(2,4-dichlorophenoxy)-2-aminobenzoic acid methyl ester (acetonitrile extracts). Bifenox was the dominant compound, particularly in the single high and repeated dose groups.

Table 2.1.2-2 Metabolite identification approach in faeces and urine in rats treated with Bifenox using HPLC

Urine sample / retention time	Relative % radioactivity measured											
	2.2-3.0		4.4-4.6		4.7-5.5 Bifenox acid		6.3-6.7 Aminobifenox acid		9.7-9.8 Bifenox		11.8-12.4 Aminobifenox	
Sex	m	f	m	f	m	f	m	f	m	f	m	f
900 mg/kg bw												
0-24 h	23.3	15.3	5.2	0	71.5	84.7	0	0	0	0	0	0
24-48 h	35	11.5	0	0	65	80.2	0	8.3	0	0	0	0
24-48 h + acid hydrolysis	28	0	23	4.6	49	95.1	0	0	0	0	0	0
90 mg/kg bw												
0-24 h	16	0	0	0	84	100	0	0	0	0	0	0
24-48 h		0		0		100		0		0		0
Faecal sample/retention time	2.1-2.8		3.6-3.7		4.3-4.7		4.9-5.5 1007		6.6-7.2 1005		9.3-9.9 Bifenox	11.7-12.19 1004
900 mg/kg bw												
0-24 h	0	0	0	0	1.2	0	0	0	1.5	0	85.4 (m) 79.6 (f)	12 (m) 20.4 (f)
24-48 h	2	0.4	0.9	0	0	0	1.8	1	6.2	2.8	63.7 (m) 68 (f)	25.4 (m) 27.9 (f)
90 mg/kg bw												
24-48 h		0		11.5		0		17.8		32.3	12.3 (f)	26.1 (f)

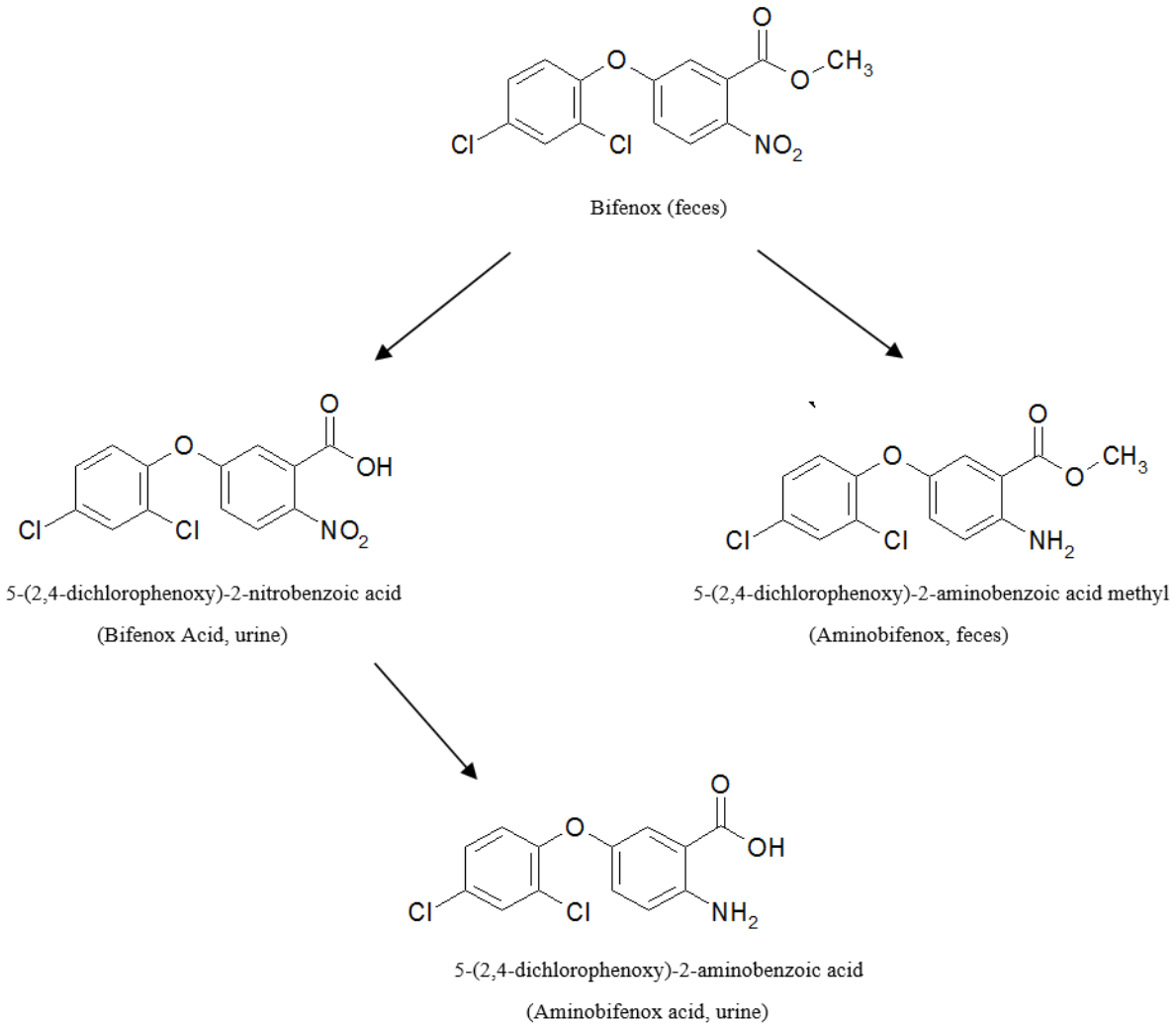
Male and female rats were dosed orally with ^{14}C-ring labelled Bifenox at 90 and 900 mg/kg bw and also after repeated dosing with non-labelled Bifenox at 90 mg/kg bw for 14 consecutive days followed on day 15 by a single oral dose of [^{14}C]-ring labelled Bifenox. The following data on excretion over 7 days were determined.

Table 2.1.2-3 Elimination of [^{14}C]-Bifenox

Elimination over 7 days after dosing				
Sex	**Treatment**	**Dose [mg/kg]**	**Urine**	**Faeces**
Males	Single oral	90	29.11%	63.41%
Females	Single oral	90	52.64%	46.24%
Males	Single oral	900	11.67%	84.90%
Females	Single oral	900	19.56%	82.46%
Males	Repeated oral	90	20.50%	78.05%
Females	Repeated oral	90	38.52%	58.04%

A marked sex difference in absorption/elimination is notable. Radioactivity detected in urine for all groups and both sexes was associated mainly with 5-(2,4-dichlorophenoxy)-2-nitrobenzoic acid. No Bifenox was detectable in urine. In faeces excreted radioactivity was associated mainly with Bifenox and 5-(2,4-dichlorophenoxy)-2-aminobenzoic acid methyl ester (acetonitrile extracts). Bifenox was the dominant compound, particularly in the single high and repeated dose groups.

Proposed metabolism for Bifenox



Residual radioactivity in tissue seven days after dosing were low and associated mainly with plasma, liver and kidney. Slightly more radioactivity was found in males and residues in single and repeated dosing at 90 mg/kg were comparable. Radioactivity in the 900 mg/kg single oral dose groups was slightly higher.

Elimination phase mean half-life values were significantly lower for males than for females at both dose levels. Males dosed at 900 mg/kg had a significantly higher ($P < 0.01$) mean half-life value than those of the low dose group. No such differences between dose groups were observed with females.

Table 2.1.2-4 Mean half-life and peak blood values

Sex	Single oral dose [mg/kg]	Mean half-life [h]	Peak blood concentration [µg/mL]
Males	90	39.14	135
Females	90	66.36	57
Males	900	55.52	185
Females	900	70.02	75

The kinetic studies revealed that males had higher blood peak values at both dose levels and peak occurred approximately 2 hours later in males.

2.1.3 Study 3 - CA 5.1.1 Absorption, distribution, metabolism and excretion by other routes

Report:	Anonymous (2016)
Title:	Bifenox: Biliary Excretion in Rats
Document No:	QB04CD
Guidelines:	OECD 417, OPPTS 870.7485
GLP	Yes

Table 2.1.3-1 Summary of ADME studies with Bifenox

Species	Dose	Test substance	Result	GLP / Guideline	Reference
New study					
In vivo, bile duct cannulated rat	90 and 900 mg/kg bw	[^{14}C]-Bifenox Batch # CFQ42846 Purity: ≥ 97%	Oral absorption 90 mg/kg bw: 30.3% male - 50.3% female Oral absorption 900 mg/kg bw: 12.9% male - 16.2% female	GLP / OECD 417	Ford, 2016

Executive Summary

The absorption and excretion of radio-labelled [^{14}C]-Bifenox after single oral doses to bile duct-cannulated male and female Sprague Dawley CD rats at 90 mg/kg bw and 900 mg/kg bw were investigated in this toxicokinetics study.

After single oral doses of [^{14}C]-Bifenox to bile duct-cannulated rats, excretion of radioactivity was rapid with more than 85% of the dose excreted within the first 24 hours post dosing. Biliary excretion accounted for ≤ 18% of the dose at the 90 mg/kg dose level and ≤ 8.0% of the dose at the 900 mg/kg dose level.

Excretion of radioactivity was predominantly via the faeces and accounted for 42.8 – 72.9% dose during 0 – 48 hours at the 90 mg/kg dose level and 89.7 – 96.5% dose at the 900 mg/kg dose level. Urinary excretion during 0 – 48 hours accounted for 13.9 – 32.0% dose at the 90 mg/kg dose level and 4.8 – 9.1% dose at the 900 mg/kg dose level. A sex difference was noted in the routes of excretion at the 90 mg/kg dose level as a higher proportion of radioactivity was eliminated in the urine in females, whilst a greater proportion of radioactivity was eliminated in the faeces of males.

The extent of absorption was assessed as the sum of total radioactivity measured in bile, urine, liver and carcass. On this basis it was estimated that the extent of absorption was 30.3% and 50.3% of the dose for males and females, respectively at the 90 mg/kg dose level and 16.2 % and 12.9% dose for males and females at the 900 mg/kg dose level. At 48 hours,

retention of radioactivity in residual carcass and tissues was low, accounting for 0.5 – 0.8% dose at the 90 mg/kg dose level and 0.1 – 0.9% at the 900 mg/kg dose level.

This metabolism study in the Sprague Dawley CD rats is acceptable and satisfies the guideline requirement for a toxicokinetic study OECD 417 and US EPA OPPTS 870.7485 in Sprague Dawley CD rats.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Name:	Bifenox
Chemical Name:	Methyl 5-(2,4-dichlorphenoxy)-2-nitrobenzoate
Lot #:	D-20140741P
Purity:	98%
Expiry date:	June 2017
Radiolabelled Material:	[dichlorphenyl-ring-U-^{14}C]-Bifenox
Lot #:	CFQ42846
Specific Activity:	3.85 MBq/mg
Radiochemical purity:	Prior to formulation ≥ 98% After formulation ≥ 97%
Expiry date:	Not specified

2. Vehicle and/or control materials

Vehicle:	0.25% (w/v) aqueous gum tragacanth (Fisher scientific, batch 1343905, expiry 16 April 2018)

3. Test animals

Species, Strain:	Rat *Rattus norvegicus*
Source:	Charles River (UK)
Strain:	Sprague-Dawley CD (albino) [Crl:CD(SD)]
Sex:	Male and female (nulliparous and non-pregnant)
Age:	9 – 13 weeks
Body weight:	264 - 320 g (males); 233 - 281 g (females)
Number of animals:	4 males (3 + 1 additional dosed animal) and 4 females (3 + 1 additional dosed animal) for low dose, 8 males (6 + 2 additional dosed animal) and 4 females (3 + 1 additional dosed animal) for high dose
Acclimatisation period:	2 days
Housing:	Prior to dosing, animals were housed individually, in solid-bottom polycarbonate cages with stainless steel lids and wood flakes grade ¾ and environmental enrichment (plastic tunnel/shelter, wooden chew-block). No rats were housed with rats of another study group or sex. After dosing, rats were housed individually in glass metabolism cages (Metabowls®), which facilitated the separate collection of urine and faeces.

4. Environmental conditions

Temperature:	22 ± 2°C
Humidity:	55 ± 15%
Photoperiod:	Alternating 12 hour light/dark

B. STUDY DESIGN AND METHODS

1. In-life dates

18. July – 25. November 2015 at Envigo CRS Limited, Cambridgeshire, UK

2. Bile duct-cannulated rats

Bile duct-cannulated Sprague-Dawley CD rats were obtained ready prepared from a commercial supplier (Charles River UK). Prior to dosing, the cannula loop was exposed and opened, flow of bile confirmed and the now separate bile duct and duodenal cannula extended with further tubing and attached to a dual-channel swivel, permitting full animal mobility within its cage. Each rat was then transferred to a glass metabolism cage for sample collection. After dosing, a solution of bile salts was infused via the duodenal cannula at a rate of 0.65 mL/hour until sacrifice.

3. Preparation of the test formulation

An aliquot of [^{14}C]-Bifenox stock solution (4.6 – 5.1 mL equivalent to 2.34 – 2.60 mg) and non-radiolabelled Bifenox (159.70 – 168.58 mg and 1797.96 – 1798.10 mg for the low and high dose levels respectively) were suspended in 0.25% (w/v) aqueous gum tragacanth after purification of the stock solution. Each formulation was prepared the day before administration.

4. Dose administration

The dose solutions were administered orally using a graduated syringe with rubber gavage tube at a nominal rate of 5 mL (Group 1) or 7.5 mL (Group 2) formulation/kg bw. The actual amount of formulation administered was determined gravimetrically by weighing the dose syringe when loaded and again after administration.

5. Dose analyses

To accurately determine and verify the quantity of radioactivity administered to each animal, three further aliquots (nominally 0.25 – 0.4 mL) of dose formulation were taken for radioassay.

The radiochemical purity of [^{14}C]-Bifenox was determined by high performance liquid chromatography (HPLC) with on-line radioactivity detection in each dose formulation prepared (post-dose).

6. Sample collection

Groups of four bile duct-cannulated rats per sex received a single low dose (Groups 1) of [^{14}C]-Bifenox, Group 2 bile duct-cannulated rats (8 male, 4 female) received a single high level dose of [^{14}C]-Bifenox by oral gavage. Bile, urine and faeces were collected into solid CO_2 cooled containers at the following intervals post administration:

Bile	0 – 3, 3 – 6, 6 – 9, 9 – 12, 12 – 24 and 24 – 48 hours
Urine and faeces	0 – 12, 12 – 24 and 24 – 48 hours

At the end of each 24 hour collection period, the interiors of the metabolism cages were rinsed with water which were drained into the urine sample. At 48 hours, the animals were sacrificed by cervical dislocation and the gastrointestinal tract and liver were removed from each carcass and retained with the remaining carcass for analysis. The interior of the metabolism cages were washed well with ethanol and distilled water after sacrifice and the washings were retained.

7. Statistical analysis

For each time separately, an assessment was made of the externally Studentised residuals to check for outliers in the dataset using software SAS, Version 9.1.3 SP 4. Residuals greater than 3 or less than -3 were considered to be potential outliers.

C. OBSERVATIONS

During the course of the study, all animals were routinely observed for behavioural changes, ill health or reactions to treatment. On the day of dosing, all animals were observed immediately after dosing, again within 2 hours after dosing

and on at least one other occasion during the working day. On all other days after dosing, all animals were observed on at least one occasion.

D. ANALYTICAL METHODS

1. Processing of samples for radioactivity measurement

For the determination of total radioactivity and concentrations of radioactivity in samples, total weights, replicate weights and liquid scintillation counting data were recorded and processed using the DEBRA automated laboratory data capture and processing system (V5.5.4.49, LabLogic Systems Ltd, Sheffield, UK).

Samples of urine, faeces, cage wash and bile were allowed to thaw and the total weights were measured. Duplicate weighed aliquots of all samples were taken for radioassay. Liver and gastrointestinal tracts (including contents) were homogenised before taking duplicate samples for radioassay after combustion. The remaining carcasses were weighed prior to digestion. Carcasses were solubilised, the weight of the digest was recorded and replicate weighed aliquots were taken for radioassay.

2. Measurement of radioactivity

Radioactivity was measured by liquid scintillation counting (LSC).

Solid samples were combusted using a sample oxidiser. Radioactivity in gross amounts of less than twice the background level was considered to be below the limit of accurate determination (limit of detection). HPLC was carried out with UV (254 nm) and radioactivity detection.

II. RESULTS AND DISCUSSION

A. DOSE LEVELS

Body weights at dosing ranged from 264 – 320 (males) and 233 – 281g (females). Doses administered ranged from 84.8 to 99.5 mg/kg (low dose level) and from 875.7 – 985.1 mg/kg (high dose level) and were in consistent with the appropriate nominal target doses levels (90 and 900 mg/kg).

The doses administered to animals 9M – 12M were considered lower than acceptable (613 – 690 mg/kg) so excretion data for these animals is not reported in the following. An additional four male rats were therefore dosed (17M – 20M). The actual body weights and dose levels of all [^{14}C]-Bifenox administered to rats are shown in **Table 2.1.3-2**.

Table 2.1.3-2 Animal body weights and doses administered

Animal number and sex	Weight at dosing [g]	Administered dose	
		[g]	[mg/kg bw]
Group 1 (low dose level, target dose 90 mg/kg bw) Specific activity: 1M – 4M = 2393 dpm/µg 5F – 8F = 3174 dpm/µg			
1M	295	1.5001	99.5
2M	305	1.5284	98.1
3M	264	1.3036	96.6
4M	274	1.3525	96.6
5F	233	1.1456	84.8
6F	250	1.2427	85.8
7F	242	1.2005	85.6
8F	281	1.4537	89.3
Group 2 (high dose level, target dose 900 mg/kg bw) Specific activity: 9M – 12M = 248 dpm/µg 13F – 16F = 338 dpm/µg			
9M	302	1.5643	639.4[a]
10M	290	1.4403	613.1[a]
11M	298	1.6652	689.8[a]
12M	306	1.5405	621.4[a]
13F	274	2.1231	882.1
14F	275	2.1152	875.7
15F	269	2.0849	882.4
16F	259	1.9989	878.6
Group 2 – additional animals (high dose level, target dose 900 mg/kg bw) Specific activity: 327 dpm/µg			
17M	314	2.3596	960.1
18M	292	2.2040	964.4
19M	320	2.3849	952.2
20M	317	2.4442	985.1

a Dose group repeated as correct dose level not achieved

B. CLINICAL SIGNS AND NECROPSY

1. Low dose level (90 mg/kg)

No adverse clinical signs were observed following dose administration.

2. High dose level (900 mg/kg)

Following dosing of the male animals, piloerection was observed up to 22 hours post dosing. During necropsy, pale patches and speckling were observed on the liver. Following dosing of the female animals, fast/irregular breathing was

observed up to 3 hours post dosing. Partially closed/ dull eyes were observed up to 4 hours and piloerection was observed up to 23 hours post dosing.

C. EXCRETION DATA LOW DOSE LEVEL (GROUP 1)

Following a single oral administration to male and female rats, biliary excretion accounted for 15.6% and 17.8% dose in males and females, respectively. Most of the biliary excretion (> 96%) occurred during 0 – 24 h post dosing, accounting for 15.0% and 17.1% of the dose in males and females, respectively. Urinary excretion accounted for a mean total of 13.9% and 32.0% dose in males and females, respectively, with faecal elimination accounting for 72.9% and 42.8% dose. Cage washings accounted for 0.7% and 1.2% of dose in males and females, respectively.

Overall excretion of radioactivity was rapid, with > 85% dose excreted during 0 – 24 hours. Radioactivity remaining in the residual carcass and tissues at 48 hours accounted for 0.5 – 0.8% of the dose. Overall recoveries were 104.0% of the dose for males and 94.4% of the dose for females.

The extent of absorption was estimated by summing the values for bile, urine, liver and carcass. On this basis it was estimated that the extent of absorption was 30.3% dose for males and 50.3% dose for females.

There was a sex-related difference in the routes of excretion, with a higher proportion of radioactivity being eliminated in the urine in females, whilst a greater proportion of radioactivity was eliminated in the faeces of males.

Excretion data are shown in **Table 2.1.3-3.** Unless otherwise stated, data are mean values. Data for animal 5F was excluded due to anomalous results compared to other animals in the same group and statistical analysis showed that this animal was an outlier. Samples from the additional animal 8F were considered and analysed instead.

Table 2.1.3-3 Excretion and retention of radioactivity following a single oral administration of [^{14}C]-Bifenox (90 mg/kg) to bile duct-cannulated rats (Group 1)

	% of dose administered (90 mg/kg bw)										
	Males Group 1					Females Group 1					
	1M	2M	3M	Mean	SD	5F[c]	6F	7F	8F	Mean	SD
Bile (hours)											
0 - 3	0.5	0.9	0.5	0.6	0.2	1.8	0.7	0.4	1.2	0.8	0.4
3 – 6	2.1	3.9	3.7	3.2	1.0	1.3	1.7	2.1	1.1	1.6	0.5
6 – 9	4.5	5.6	3.9	4.7	0.9	1.2	4.2	4.8	1.7	3.6	1.6
9 – 12	6.2	2.9	1.7	3.6	2.3	1.1	4.6	5.3	3.0	4.3	1.2
12 – 24	5.0	2.4	1.3	2.9	1.9	1.1	6.5	5.2	8.9	6.9	1.9
24 - 48	0.9	0.4	0.5	0.6	0.3	1.5	0.5	0.4	1.0	0.6	0.3
Total bile	19.2	16.1	11.6	**15.6**	3.8	8.0	18.2	18.2	16.9	**17.8**	0.8
Urine (hours)											
0 – 12	5.3	10.9	14.1	10.1	4.5	66.2	28.7	23.2	31.8	27.9	4.4
12 – 24	3.0	3.3	2.2	2.8	0.6	5.5	2.8	2.6	4.4	3.3	1.0
24 - 48	1.4	0.9	0.6	1.0	0.4	0.7	0.9	0.5	1.2	0.9	0.4
Total urine	9.7	15.1	16.9	**13.9**	3.7	72.4	32.4	26.3	37.4	**32.0**	5.6
Faeces (hours)											
0 – 12	13.3	66.8	75.2	51.8	33.6	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
12 – 24	46.3	14.5	1.3	20.7	23.1	21.7	34.4	47.9	28.0	36.8	10.2
24 – 48	0.3	0.1	1.0	0.5	0.5	0.2	2.5	2.7	13.0	6.1	6.0
Total faeces	59.9	81.4	77.5	**72.9**	11.5	21.9	36.9	50.6	41.0	**42.8**	7.0
Samples after necropsy											
Cage wash	1.3	0.5	0.3	0.7	0.5	0.7	0.3	0.3	3.2[b]	1.2	1.7
Liver	0.1	0.1	< 0.1	0.1	0.1	< 0.1	0.1	0.1	0.1	0.1	< 0.1
G.I.T. and contents	< 0.1	ND	0.1	< 0.1	0.1	ND	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Residual carcass	1.6	0.4	0.2	0.7	0.8	0.3	0.4	0.3	0.6	0.4	0.2
Total recovery	91.8	113.6	106.6	104.0	11.1	103.3	88.3	95.8	99.2	94.4	5.6
Total absorption[a]	30.6	31.7	28.7	**30.3**	1.5	80.7	51.1	44.9	55.0	**50.3**	5.1

SD standard deviation
ND Not detected
G.I.T. Gastrointestinal tract
a Sum of values for bile, urine, liver and residual carcass
b Results from cage wash 1 only, cage wash 2 sample lost prior to analysis
c Data from rat 5F not included in mean and SD calculations due to anomalous results

D. EXCRETION DATA HIGH DOSE LEVEL (GROUP 2)

Following a single oral administration to male and female rats, biliary excretion accounted for 8.0% and 7.0% dose in males and females, respectively. Most of the biliary excretion (> 91%) occurred during 0 – 24 hours post dose, accounting for 7.7% and 6.4% dose in males and females, respectively. Urinary excretion accounted for a mean total of 4.8% and 9.1% dose in males and females, respectively, with faecal elimination accounting for 89.7% and 96.5% dose. Cage washings accounted for 0.3% and 0.1% of dose in males and females, respectively.

Overall excretion of radioactivity was rapid, with > 98% dose excreted during 0 – 24 hours. Radioactivity remaining in the residual carcass and tissues at 48 hours accounted for 0.1 – 0.9% dose. Overall recoveries were 102.9% dose for males and 113.6% dose for females.

The extent of absorption was estimated by summing the values for bile, urine, liver and carcass. On this basis it was estimated that the extent of absorption was 12.9% dose for males and 16.2% dose for females.

Excretion data are shown in **Table 2.1.3-4**. Unless otherwise stated, data discussed in this section are mean values.

Table 2.1.3-4 Excretion and retention of radioactivity following a single oral administration of [^{14}C]-Bifenox (900 mg/kg) to bile duct-cannulated rats (Group 2)

	% of dose administered (900 mg/kg bw)									
	Males Group 2					Females Group 2				
	17M	18M	19M	Mean	SD	13F	14F	15F	Mean	SD
Bile (hours)										
0 - 3	0.1	0.2	0.3	0.2	0.1	0.1	0.1	0.1	0.1	< 0.1
3 – 6	0.5	0.6	0.8	0.6	0.2	0.6	0.1	0.5	0.4	0.3
6 – 9	1.2	1.0	1.9	1.4	0.5	1.1	0.3	1.1	0.8	0.5
9 – 12	1.8	0.9	2.6	1.8	0.9	1.7	0.7	2.1	1.5	0.7
12 – 24	4.1	0.9	6.1	3.7	2.6	3.8	2.1	4.9	3.6	1.4
24 - 48	0.3	0.1	0.5	0.3	0.2	0.3	1.0	0.5	0.6	0.4
Total bile	8.0	3.7	12.2	**8.0**	4.3	7.6	4.3	9.2	**7.0**	2.5
Urine (hours)										
0 – 12	3.2	3.8	2.7	3.2	0.6	7.5	5.6	5.1	6.1	1.3
12 – 24	1.3	0.8	1.6	1.2	0.4	5.1	1.1	1.5	2.6	2.2
24 - 48	0.3	0.2	0.5	0.3	0.2	0.5	0.5	0.3	0.4	0.1
Total urine	4.8	4.8	4.8	**4.8**	< 0.1	13.1	7.2	6.9	**9.1**	3.5
Faeces (hours)										
0 – 12	2.0	57.8	1.0	20.3	32.5	27.6	< 0.1	21.7	16.4	14.5
12 – 24	83.6	32.0	82.2	65.9	29.4	65.9	90.5	72.6	76.3	12.7
24 – 48	2.0	1.0	7.6	3.5	3.6	1.4	7.7	2.1	3.7	3.5
Total faeces	87.6	90.8	90.8	**89.7**	1.8	94.9	98.2	96.4	**96.5**	1.7
Samples after necropsy										
Cage wash	< 0.1	0.6	0.2	0.3	0.3	0.1	< 0.1	0.2	0.1	0.1
Liver	< 0.1	< 0.1	0.1	< 0.1	0.1	< 0.1	< 0.1	0.1	< 0.1	0.1
G.I.T. and contents	ND	ND	< 0.1	< 0.1	< 0.1	1.6	0.6	0.1	0.8	0.8
Residual carcass	0.1	< 0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	< 0.1
Total recovery	100.5	99.9	108.2	102.9	4.6	117.4	110.4	113.0	113.6	3.5
Total absorption[a]	12.9	8.5	17.2	**12.9**	4.4	20.8	11.6	16.3	**16.2**	4.6

SD standard deviation
ND Not detected
G.I.T. Gastrointestinal tract
a Sum of values for bile, urine, liver and residual carcass

III. CONCLUSION

After single oral doses of [^{14}C]-Bifenox to bile duct-cannulated rats at dose levels of 90 and 900 mg/kg bw, excretion of radioactivity was rapid with more than 85% of the dose excreted within the first 24 hours post dose. Biliary excretion accounted for ≤ 17.8% dose at the 90 mg/kg dose level and ≤ 8.0% dose at the 900 mg/kg dose level.

Excretion of radioactivity was predominantly via the faeces and accounted for 42.8 – 72.9% dose during 0 - 48 hours at the 90 mg/kg dose level and 89.7 – 96.5% dose at the 900 mg/kg dose level. Urinary excretion during 0 – 48 hours accounted for 13.9 – 32.0% dose at the 90 mg/kg dose level and 4.8 – 9.1% dose at the 900 mg/kg dose level. A sex

difference was noted in the routes of excretion at the 90 mg/kg dose level as a higher proportion of radioactivity was eliminated in the urine in females, whilst a greater proportion of radioactivity was eliminated in the faeces of males.

At 48 hours, retention of radioactivity in residual carcass and tissues was low, accounting for 0.5 – 0.8% dose at the 90 mg/kg dose level and 0.1 – 0.9% at the 900 mg/kg dose level.

The extent of absorption was assessed as the sum of total radioactivity measured in bile, urine, liver and carcass. On this basis, it was estimated that the extent of absorption was 30.3% and 50.3% dose for males and females respectively at the 90 mg/kg dose level and 16.2 % and 12.9% dose for males and females respectively at the 900 mg/kg dose level.

Conclusions for setting the AOEL of Bifenox

The EU agreed AOEL of Bifenox (0.125 mg/kg bw) is corrected for limited oral absorption and bioavailability (estimated 25%) as concluded in the EFSA peer review report of Bifenox (2007).

Rate and extent of oral absorption/systemic bioavailability:	Based on urinary excretion: 29% and 53% in male and female rats.		
Acceptable Operator Exposure Level (AOEL):	0.125 mg/kg bw/day (Agreed EU endpoint)	Rabbit, developmental, supported by the 2-generation study in rats	400*

* Including correction for limited oral absorption/bioavail-ability (25 %)

The results of the present study have been considered for the setting of the AOEL for the renewal of the approval of Bifenox under Regulation (EC) 1107/2009. The absorption values from a study that was already submitted in the context of the inclusion of the active substance Bifenox in Annex I of the Council Directive 91/414/EEC, which was considered for the determination of the oral absorption have been compared to the new absorption values as demonstrated in **Table 2.1.3-5**.

Table 2.1.3-5 Absorption values considered for setting the AOEL of Bifenox

Sex	Treatment	Dose [mg/kg]	Koundakjian et al., 1986*	Ford, 2016**
			Absorption from dose administered	
Males	Single oral	90	29.11%	30.3%
Females	Single oral	90	52.64%	50.3%
Males	Single oral	900	11.67%	12.9%
Females	Single oral	900	19.56%	16.2%
Males	Repeated oral	90	20.50%	n.a.
Females	Repeated oral	90	38.52%	n.a.

* Based on urinary excretion
** Sum of values for bile, urine, liver and residual carcass
n.a. Single oral doses were administered only

There is no significant difference when comparing the absorption values is rats from both studies (Koundakjian et al., 1986 and Ford, 2016) which need to be considered for the determination of the oral absorption.

It is concluded that the rate of oral absorption from Bifenox is confirmed to be 25% based on the results of this biliary excretion study. Therefore, no refined correction for oral absorption of the AOEL is necessary.

The AOEL remains at 0.125 mg/kg bw/day.

3 HEALTH HAZARDS

3.1 Acute toxicity - oral route

3.1.1 Animal data

3.1.1.1 Study 1 - CA 5.2.1 Acute oral toxicity

Study reference

Report:	Anonymous (1985a)
Title:	Acute oral toxicity study on rats
Document No:	Bio/dynamics, Inc. 5799-85
Guidelines:	EPA 81-1 870.1100
GLP	Yes

Detailed study summary and results

This study does not provide a statement of GLP but the study report claims GLP compliance. The study has been performed in accordance with EPA Guideline 81-1 870.1100 and an internal quality assurance statement is provided. Furthermore, the study was accepted in the DAR (2006). In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool) has been used for evaluation. Consequently, this study is categorized into Klimisch category 1: reliable without restriction. Therefore, it is concluded that this study is valid, even without a statement of GLP and is considered for classification.

Test substance, Test animals, Administration/exposure

Bifenox (lot no. 3123142017, purity 97%) was administered orally by gavage, to five male and five female Sprague-Dawley rats, at a level of 5000 mg/kg bw. Signs of toxicity and body weights were recorded up to 14 days after dosing. Gross pathological examinations were performed on all main study animals.

Results

No mortality was recorded during the study. The majority of the animals showed expected body weight gain throughout the study. Clinical signs included faecal staining, soft stool and hypoactivity (1-2 animals) 24 hours after dosing. 2 days after oral administration, some animals showed alopecia of abdomen, chest and/or hind leg. Reduced food consumption was observed in some cases. At necropsy, some animals showed red foci and discoloration of lungs.

The results on bodyweight gain and mortality are summarized in T**able 3.1.1-1.**

Table 3.1.1-1 Summary of body weight gains and mortalities at 5000 mg/kg Bifenox

Animal No.	Body weight gain [g]					Mortality (day)
	Pre-test	Day 7	Change*	Day 14	Change*	
Males						
2066	256	285	29	327	71	14**
2072	264	307	43	365	101	14**
2076	262	263	1	329	67	14**
2081	255	265	10	346	91	14**
2096	250	250	0	316	66	14**
Females						
2108	242	260	18	270	28	14**
2115	230	246	16	261	31	14**
2119	242	270	28	290	48	14**
2126	235	259	24	280	45	14**
2130	236	250	14	275	39	14**

* At terminal sacrifice – not substance related
** Change from pre-fasted weight

Conclusion

The acute oral median lethal dose (LD_{50}) of Bifenox in rats was greater than 5000 mg/kg body weight. Based on the acute oral LD_{50} value, Bifenox does not require classification and labelling according to Regulation (EC) 1272/2008.

3.1.1.2 Study 2 - CA 5.2.1 Acute oral toxicity

Study reference

Report:	Anonymous (1978)
Title:	Acute Toxicity Study in mice Bifenox (MCTR-126-78)
Document No:	Litton Bionetics, 20982
Guidelines:	Not stated
GLP	No

Detailed study summary and results

This study does not provide a statement of GLP, not even a statement on internal quality control. Reporting is very brief and no information on test item purity is given. Furthermore, this study was not performed in accordance with any guidelines and it was not accepted in the DAR (2006).

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool) has been used for evaluation. Consequently, this study is categorized into Klimisch category 3: not reliable. Therefore, this study is judged not to be valid and is not considered for classification.

This study does not fully confirm to the directive EEC 92/69 method B1 or GLP and it provides only incomplete data for body weight, clinical signs and necropsy findings.

Test substance, Test animals, Administration/exposure

Bifenox was suspended in corn oil and administered orally by gavage, to mice (5 per dose-group and sex). The dose groups were between at 0, 316, 1000, 3160, 10000 mg/kg bw. Animals were observed for clinical signs of toxicity up to 14 days after dosing. Body weights were recorded at the beginning and at the end of the study. Gross pathological examinations were performed at the end of the observation period.

Results

On day two, mortality was observed at 1000 mg/kg bw in 1/5 males. At a dose level of 3160 mg/kg bw, 3/5 males and 3/5 females died on day 1, 2/5 males and 2/5 females died on day 2. At a dose level of 10000 mg/kg bw 3/5 males and 4/5 females died on day 1, 1/5 males and 1/5 females on day 2. There was no significant change in body weight gain compared to normal. Clinical signs included inactivity, unsteady gait and shivering. During necropsy, no gross lesions were observed. The results on mortality are summarised in **Table 3.1.1-2.**

Table 3.1.1-2 Mortalities in the acute oral toxicity study in mice

Group	Dose (mg/kg)	Mortalities in males	Mortalities in females
1	0	0/5	0/5
2	316	0/5	0/5
3	1000	1/5	0/5
4	3160	5/5	5/5
5	10000	4/5	5/5

Based on deaths occurring in the 14 days of treatment and using a modification of the method of Horn (Biometrics. 12. p. 311, 1956), LD50 values of 1540 mg/kg (95% confidence limits: 833-2850) and 1780 mg/kg (95% confidence limits: not determined due to steep response slope) were calculated for male and female mice, respectively.

Signs of toxicity judged to be related to treatment included inactivity, unsteady gait and shivering.

Necropsy findings among the surviving animals showed no gross lesions. Among mice that died, gas in the stomach and intestines was noted.

Following the oral administration of graded doses of the test compound to fasted young mice, LD_{50} values of 1540 mg/kg and 1780 mg/kg were calculated for males and females, respectively. The gastrointestinal tract may be a possible site of toxicity.

3.1.2 Human data

No human data available on the acute oral toxicity of Bifenox. No clinical cases or poisoning incidents were reported.

3.1.3 Other data

No other data available on the acute oral toxicity of Bifenox.

3.2 Acute toxicity - dermal route

3.2.1 Animal data

3.2.1.1 Study 1 - CA 5.2.2 Acute dermal toxicity

Study reference

Report:	Anonymous (1985b)
Title:	Acute dermal toxicity study in rabbits
Document No:	Bio/dynamics, Inc. 5800-85
Guidelines:	EPA 81-2 870.1200
GLP	Yes

Detailed study summary and results:

This study does not provide a statement of GLP but the study report claims GLP compliance. The study has been performed in accordance with EPA Guideline 81-1 870.1200 and an internal quality assurance statement is provided. Furthermore, the study was accepted in the DAR (2006).

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool) has been used for evaluation. Consequently, this study is categorized into Klimisch category 1: reliable without restriction.

Therefore, it is concluded that this study is valid and it is considered for classification of Bifenox.

Test substance, Test animals, Administration/exposure

A single dose of Bifenox (lot no. 3123142017, purity 97%) was applied to the clipped abdominal skin of male and female rats (5 animals/sex), at a dose level of 2000 mg/kg bw. The test material was moistened with saline 0.9% and the application site was covered with gauze and an impervious plastic sleeve. After 24 hours, the gauze and plastic sleeve was removed and the application site was washed out with water. All animals were observed for clinical signs for at least 14 days after treatment and were necropsied at the end of the observation period.

Results

There was no mortality. Most of the animals were free of significant signs although a single occurrence of ocular discharge was seen, as were occasional observations of nasal discharge and food consumption decrease. No abnormalities were seen at the majority of animals during necropsy.

The acute dermal median lethal dose (LD_{50}) of Bifenox in rabbits was greater than 2000 mg/kg body weight. Based on the acute dermal LD_{50} value, Bifenox does not require classification and labelling according to Regulation (EC) 1272/2008.

3.2.2 Human data

No human data available on the acute dermal toxicity of Bifenox. No clinical cases or poisoning incidents were reported.

3.2.3 Other data

No other data available on the acute dermal toxicity of Bifenox.

3.3 Acute toxicity - inhalation route

3.3.1 Animal data

3.3.1.1 Study 1 - CA 5.2.3 Acute inhalation toxicity

Study reference

Report:	Anonymous (1985)
Title:	An acute inhalation toxicity study of Bifenox (technical) in the rat
Document No:	Bio/dynamics, Inc. 85-7809
Guidelines:	EPA 81-3, EC Directive 92/69/EEC, 93/21/EEC B.2 and OECD 403
GLP	Yes

Detailed study summary and results:

This study does not provide a statement of GLP but the study report claims GLP compliance. In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool) has been used for evaluation. Consequently, this study is categorized into Klimisch category 1: reliable without restriction. Therefore, it is concluded that this study is valid and it is considered for classification of Bifenox.

Test substance, Test animals, Administration/exposure

Bifenox technical (lot no. 3123142017, purity 97%) was administered as a single four-hour exposure to five CD Sprague-Dawley rats/sex/dose. The dust atmosphere concentration was at 0.91 mg/L, which was the maximum attainable exposure concentration with a MMAD of 2.7 µm and a SGD of 1.6.

Results

No mortality was observed throughout study performance. Clinical signs included activity and white material on fur, were exhibited during exposure. Upon removal from the chamber and during the 2-hour post-exposure observation period, increased secretory response, moist rales, yellow/brown ano-genital staining and soft stool were exhibited by the test animals. During the first days of test week 1, rats exhibited yellow ano-genial staining and slightly increased secretory responses. There were no significant changes in body weight gain. During necropsy, no compound related findings were observed.

The acute (4-hour) inhalation LC_{50} for rats exposed to Bifenox was greater than 0.91 mg/L (LD_{50} of 245 mg/kg bw). Based on the acute inhalation LC_{50} value, Bifenox does not require classification and labelling according to Regulation (EC) 1272/2008.

3.3.2 Human data

No human data available on the acute inhalation toxicity of Bifenox. No clinical cases or poisoning incidents were reported.

3.3.3 Other data

No other data available on the acute inhalation toxicity of Bifenox.

3.4 Skin corrosion/irritation

3.4.1 Animal data

3.4.1.1 Study 1 - CA 5.2.4 Skin irritation

Study reference

Report:	Anonymous (1985c)
Title:	Primary dermal irritation study in rabbits
Document No:	Bio/dynamics, Inc. 5801-85
Guidelines:	EPA 81-5 870.2500
GLP	Yes

Detailed study summary and results:

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool) has been used for evaluation. Consequently, this study is categorized into Klimisch category 1: reliable without restriction. Therefore, it is concluded that this study is valid and it is considered for classification of Bifenox.

Test substance, Test animals, Administration/exposure

Bifenox (lot no. 3123142017, purity 97%) was applied as a single 0.5 g dermal dose to three New Zealand White rabbits/sex. The day after application, the back hair was clipped. The test substance moistened with 0.9% saline, applied and covered with a gauze and tape for 4 hours. After removal of the patch, residual test substance was washed out. Non-treated site served as control. The area was examined at 1, 24, 48 and 72 hours after the removal of the patch and scored according to Draize (1959).

Results

Bifenox was essentially non-irritating to the skin of rabbits. The only irritation noted was very slight erythema in one animal at 0.5 h. **Table 3.4.4-1** shows the results of the scoring for Erythema and Oedema according to Draize.

Table 3.4.1-1 Dermal irritation responses in rabbits according to the Draize scheme

Animal	Erythema score						Oedema score					
No.	1	2	3	4	5	6	1	2	3	4	5	6
Sex	m	m	m	f	f	f	m	m	m	f	f	f
after 30 min.	0	0	0	0	0	1	0	0	0	0	0	0
24 h	0	0	0	0	0	0	0	0	0	0	0	0
48 h	0	0	0	0	0	0	0	0	0	0	0	0
72 h	0	0	0	0	0	0	0	0	0	0	0	0
Irritation Index*	0						0					

* (Mean scores 24 – 72 h)

Based on mean skin irritation scores 24 to 72 hours after removal of test substance, Bifenox does not require classification and labelling according to Regulation (EC) 1272/2008.

3.4.2 Human data

No human data available on skin irritating properties of Bifenox. No clinical cases were reported.

3.4.3 Other data

No other data available on skin irritating properties of Bifenox.

3.5 Serious eye damage/eye irritation

3.5.1 Animal data

3.5.1.1 Study 1 - CA 5.2.5 Eye irritation

Study reference

Report:	Anonymous (1985d)
Title:	Eye irritation study in Rabbits - EPA
Document No:	Bio/dynamics, Inc. 5802-85
Guidelines:	EPA 81-4 870.2400
GLP	Yes

Detailed study summary and results:

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool) has been used for evaluation. Consequently, this study is categorized into Klimisch category 1: reliable without restriction. Therefore, it is concluded that this study is valid and it is considered for classification of Bifenox.

Test substance, Test animals, Administration/exposure

A single dose of 29.7 mg (equivalent to 0.1 mL) Bifenox (lot no. 3123142017, purity 97%) was administered into the elevated lower lid of the right eye of five male and four female New Zealand white rabbits. Twenty seconds after the test substance was administered, both eyes of three rabbits were washed for one minute with lukewarm water. At approximately 24 h, the treated eyes of the remaining six animals were rinsed to remove any residual test material. The animals were observed for the signs of clinical toxicity or health conditions twice daily. The treated eyes of the rabbits were observed and scored for ocular irritation approx. 1, 24, 48, and 72 hours and 7 days after instillation of the test material. Corneal injury was assessed using fluorescein dye on all rabbits at the 24 hours observation interval and at each subsequent observation until there was no stain retention for two observations. Irritation was graded and scored according to the Draize technique.

Results

Slight conjunctival irritation (redness, chemosis, discharge) and iridial changes were noted in all treated eyes at one hour. No corneal opacity or ulceration was observed. All nine animals were free of ocular irritation within 24 h to 7 days after instillation of the test article. Comparable results were obtained for washed and unwashed eyes.

Table 3.5.1-1 Eye irritation in the rabbit, individual ocular scores

Animal No.		Scores after treatment *					Mean scores (24 - 72 h)	Reversible (day)
		1 h	7 h	24 h	48 h	72 h		
1**	Corneal opacity	0	0	0	0	0	0.0	3
	Iritis****	+	0	0	0	0	0.0	
	Chemosis conjunctivae	1	1	1	0	0	0.3	
	Redness conjunctivae	1	1	1	1	0	0.7	
2**	Corneal opacity	0	0	0	0	0	0	3
	Iritis****	+	0	0	0	0	0	
	Chemosis conjunctivae	1	1	1	0	0	0.3	
	Redness conjunctivae	1	1	1	1	0	0.7	
3**	Corneal opacity	0	0	0	0	0	0.0	0
	Iritis****	+	0	0	0	0	0.0	
	Chemosis conjunctivae	1	0	0	0	0	0.0	
	Redness conjunctivae	1	0	0	0	0	0.0	
4**	Corneal opacity	0	0	0	0	0	0.0	7
	Iritis****	+	0	0	0	0	0.0	
	Chemosis conjunctivae	1	1	1	0	0	0.3	
	Redness conjunctivae	1	2	2	1	1	1.3	
5**	Corneal opacity	0	0	0	0	0	0.0	2
	Iritis****	+	+	0	0	0	0.0	
	Chemosis conjunctivae	1	1	1	0	0	0.3	
	Redness conjunctivae	1	1	1	0	0	0.3	
6**	Corneal opacity	0	0	0	0	0	0.0	3
	Iritis****	1	0	0	0	0	0.0	
	Chemosis conjunctivae	1	1	1	0	0	0.3	
	Redness conjunctivae	1	1	1	0	0	0.3	
7***	Corneal opacity	0	0	0	0	0	0.0	0
	Iritis****	+	0	0	0	0	0.0	
	Chemosis conjunctivae	1	0	0	0	0	0.0	
	Redness conjunctivae	1	0	0	0	0	0.0	
8***	Corneal opacity	0	0	0	0	0	0.0	7
	Iritis****	0	0	0	0	0	0.0	
	Chemosis conjunctivae	1	1	1	0	0	0.3	
	Redness conjunctivae	1	1	1	1	1	1.0	
9***	Corneal opacity	0	0	0	0	0	0.0	0
	Iritis****	0	0	0	0	0	0.0	
	Chemosis conjunctivae	1	0	0	0	0	0.0	
	Redness conjunctivae	1	0	0	0	0	0.0	

* scores in the range of 0 to 4 for cornea opacity and chemosis, 0 to 3 for redness of conjunctivae and 0 to 2 for iritis
** unwashed / *** washed
**** + slight deepening of the rugae or slight hyperaemia, not included in Draize grading system

Instillation of Bifenox technical into the eyes of New Zealand white rabbits produced mild and reversible ocular irritation. Based on mean eye irritation scores 24 to 72 hours after removal of test substance, Bifenox does not require classification and labelling according to Regulation (EC) 1272/2008.

3.5.2 Human data

No human data available on eye irritating properties of Bifenox. No clinical cases were reported.

3.5.3 Other data

No other data available on eye irritating properties of Bifenox.

3.6 Respiratory sensitisation

3.6.1 Animal data

There are no experimental data and no observations or indications for respiratory sensitisation.

3.6.2 Human data

There are no experimental data and no observations or indications for respiratory sensitisation.

3.6.3 Other data

There are no experimental data and no observations or indications for respiratory sensitisation.

3.7 Skin sensitisation

3.7.1 Animal data

3.7.1.1 Study 1 - CA 5.2.6 Skin sensitization

Study reference

Report:	Anonymous (2001)
Title:	Examination of Bifenox in the skin sensitisation test in guinea pigs according to Magnusson and Kligman (maximization test)
Document No:	Laboratory of Pharmacology and Toxicology KG, 14261/01
Guidelines:	OECD 406, EC B.06 Dir. 67/548/EEC V
GLP	Yes

Detailed study summary and results:

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool) has been used for evaluation. Consequently, this study is categorized into Klimisch category 1: reliable without restriction. Therefore, it is concluded that this study is valid and it is considered for classification of Bifenox.

Test substance, Test animals, Administration/exposure

The dermal sensitisation of Bifenox (batch # 0546/7, purity 98.2%) was evaluated by means of the Magnusson and Kligman Maximization test in 15 female Dunkin-Hartley guinea pigs (10 test and 5 control animals). Concentrations used were determined from a series of "sighting tests" to determine the highest non-irritating concentrations for the intradermal and topical application route. For the intradermal induction phase, a row of three injections (0.1 mL) was made on each side of the midline of a shaved area of skin on the shoulder region:

i) 0.1 mL Freund's complete adjuvant (FCA) plus saline (0.9% NaCl) in a ratio of 1:1

ii) 0.1 mL Bifenox (w/v) in sesame oil (5%)

iii) 0.1 mL Bifenox (w/v) in sesame oil (5%) and FCA

A 5% Bifenox (w/w) in sesame oil was applied by intracutaneous induction. The animals received 2 injections into the shoulder region on day 0. On day 6, as the test substance was irritating to the skin of the test animals, it was not necessary to induce irritation by sodium laurylsulfate in vaseline. After 7 days, animals were exposed again, using the patch-test technique (induction). Challenge was performed two weeks after topical induction. The flanks of test animals were shorn and the test material applied on filter paper for a 24-h exposure. 24 hours after removal of the filter paper, skin reactions were recorded, followed by another observation after 48 hours. The skin reaction results from the first stage were evaluated at 25 and 48 hours, of the second stage at 49 and 72 hours after begin of exposure.

Control animals were treated in the same way, except that Bifenox was excluded from the induction phases. Positive control group were treated with Benzocaine at 2% w/v in a study conducted previously.

Results

Intracutaneous injections of 0.1 mL of a 5% mixture of Bifenox in sesame oil resulted in discrete or patchy erythema in all treated animals after 25 and 48 hours. Topical application of 2 mL of a 30% mixture of Bifenox in sesame oil result in discrete or patchy erythema or moderate and confluent erythema in all treated animals after 49 and 72 hours. No skin irritations were noted after challenge with the 5% mixture of Bifenox in sesame oil. At no time were adverse reactions noted at the vehicle control sites in test animals or at any sites of control animals.

Under the conditions of the Magnusson Kligman test, Bifenox did not exhibit skin-sensitizing properties in guinea pigs and was considered to be non-sensitizing.

According to the criteria laid down in Regulation (EC) 1272/2008, the test substance Bifenox does not require classification for skin sensitizing properties.

3.7.1.2 Study 2 - CA 5.2.6 Skin sensitization

Study reference

Report:	Anonymous (1985)
Title:	Delayed contact hypersensitivity in the guinea-pig with Bifenox, Technical
Document No:	Huntingdon Research Centre Ltd. 84676D/RNP
Guidelines:	OECD 406
GLP	Yes

Detailed study summary and results:

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool) has been used for evaluation. Consequently, this study is categorized into Klimisch category 1: reliable without restriction. Therefore, it is concluded that this study is valid and it is considered for classification of Bifenox.

Test substance, Test animals, Administration/exposure

10 female Hartley guinea pigs were exposed to Bifenox technical (lot no. 353-12-1, purity 98%). 10 animals were used as control group. A preliminary test was performed to identify a slightly irritating concentration. The animals received once weekly during a 3-week induction period an application of 0.5 mL test article (50% w/v in acetone) to approximately 2 cm^2 of clipped skin on the left shoulder region for 6 hours. Thereafter, the dressing was removed. The resulting dermal

reactions were assessed 24 hours later for erythema and oedema according to the Draize scale. Control animals were treated similarly without test substance. Following a rest of 2 weeks, a challenge cutaneous application of 0.5 mL test article (50% w/v in acetone) to the clipped skin (2 cm^2) of the right flank for 6 hours. Cutaneous reactions were evaluated 24, 48 and 72 hours after challenge application.

Results

Under the conditions of the Buehler test, Bifenox did not exhibit skin-sensitizing properties in guinea pigs and was considered to be non-sensitizing.

According to the criteria laid down in Regulation (EC) 1272/2008 the test substance Bifenox does not require classification for skin sensitizing properties.

3.7.2 Human data

No human data available on skin sensitizing properties of Bifenox. No sensitization cases were reported.

3.7.3 Other data

No other data available on skin sensitizing properties of Bifenox.

3.8 Germ cell mutagenicity

3.8.1 *In vitro* data

The potential of genotoxic effects from Bifenox has been evaluated based on *in vitro* and *in vivo* studies conducted in different species and cell types. None of the studies investigated resulted in a genotoxic potential from the active substance Bifenox.

3.8.1.1 Study 1 - CA 5.4.1 *In vitro* genotoxicity/mutagenicity

Study reference

Report:	KCA 5.4.1/01, Anonymous (2015)
Title:	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium* and *Escherichia coli*) with Bifenox technical
Document No:	160130, 90019663
Guidelines:	OECD 471, EC 440/2008 B. 13/14, EPA, OPPTS 870.5100, 712-C-98-247
GLP	Yes

Detailed study summary and results:

Test substance and procedure

The purpose of this study was to establish the potential of Bifenox (D-20140741) to induce gene mutations in bacteria by means of a *S. typhimurium* and *E. coli* reverse mutation assay.

At least six different concentrations of the test item are tested with approximately half log (i.e. √10) intervals between test points for an initial test. More narrow spacing between dose levels may be appropriate when a dose response is

investigated. For soluble, non-toxic test compounds the maximum test concentration is 5 mg/plate or 5 µL/plate. To validate the test, reference mutagens are tested in parallel to the test item.

Results

Mutation factors from the control plates (solvent control, 4-NOPD, NaN_3, 2-AA and B[a]P) showed the expected results, validating the assay.

In the pre-experiment, the number of revertants was only slightly affected by the test substance Bifenox. There was no significant diminution or change of revertant colonies (≤ 0.5) in comparison to the solvent control (MF = 1). Therefore, no cytotoxicity from the active substance Bifenox was revealed up to 5000 µg/plate, which was chosen to be the highest concentration for the main experiment.

The results from the main experiment plate-incorporation test are demonstrated in **Table 3.8.1-1** and **Table 3.8.1-2.**

Table 3.8.1-1 Results from the plate-incorporation test – without metabolic activation (-S9)

Treatment	Dose [µg/plate]	TA 98		TA 100		TA 1535		TA 1537		E. coli WP2 uvrA	
		RC	MF	RC	MF	RC	MF	RC	MF	RC	MF
Aqua dest.		24 ± 1.0	1.0	102 ± 11.8	1.1	5 ± 1.0	0.7	5 ± 2.0	0.9	33 ± 16.7	1.3
DMSO		24 ± 0.6	1.0	90 ± 4.4	1.0	7 ± 3.0	1.0	6 ± 1.5	1.0	25 ± 6.1	1.0
Test Item	10.0	28 ± 6.1	1.2	77 ± 11.5	0.9	17 ± 4.0	2.4	5 ± 1.2	0.8	25 ± 4.6	1.0
	31.6	27 ± 7.5	1.1	82 ± 6.0	0.9	17 ± 2.6	2.4	4 ± 2.3	0.6	38 ± 7.2	1.5
	100	25 ± 4.4	1.1	64 ± 8.4	0.7	9 ± 1.5	1.2	6 ± 2.1	1.0	29 ± 4.6	1.2
	316	31 ± 2.5	1.3	88 ± 14.4	1.0	18 ± 6.7	2.6	4 ± 0.6	0.8	49 ± 6.1	2.0
	1000	30 ± 8.4	1.3	96 ± 9.6	1.1	7 ± 2.1	1.0	11 ± 0.6	1.9	30 ± 3.0	1.2
	2500	20 ± 2.0 P	0.9	96 ± 3.6 P	1.1	7 ± 2.3 P	1.0	8 ± 1.2 P	1.4	26 ± 5.7 P	1.0
	5000	36 ± 10.6 P	1.5	114 ± 13.5 P	1.3	6 ± 4.2 P	0.9	12 ± 4.7 P	2.1	27 ± 3.6 P	1.1
4-NOPD*		431 ± 25.7	18.2	-	-	-	-	69 ± 4.0	12.2		
NaN_3*		-	-	792 ± 43.0	8.8	320 ± 84.1	45.7	-	-		
MMS*		-	-	-	-	-	-	-	-	361 ± 20.4	2.7

RC Revertant colonies: mean ± Standard deviation
MF Mutation factor: mean revertants (test item) / mean revertants (vehicle control)
* Positive control materials: NaN_3 (sodium azide), TA 100 (10 µg/plate), TA1535 (10 µg/plate), 4-NOPD (4-nitro-o-phenylene-diamine), TA 98 (10 µg/plate), TA 1537 (40 µg/plate), MMS (Methylmethanesulfonate), E. coli WP2 uvrA (1 µL)
P Precipitation

Table 3.8.1-2 Results from the plate-incorporation test – with metabolic activation (+S9)

Treatment	Dose [µg/plate]	TA 98		TA 100		TA 1535		TA 1537		E. coli WP2 uvrA	
		RC	MF	RC	MF	RC	MF	RC	MF	RC	MF
Aqua dest.		32 ± 6.2	1.1	89 ± 6.7	1.1	8 ± 4.6	0.6	4 ± 12	0.5	48 ± 2.0	1.1
DMSO		30 ± 4.2	1.0	81 ± 16.8	1.0	13 ± 4.9	1.0	7 ± 0.6	1.0	45 ± 10.5	1.0
Test Item	10.0	35 ± 8.7	1.2	108 ± 8.7	1.3	10 ± 4.0	0.7	9 ± 2.3	1.2	39 ± 8.5	0.9
	31.6	28 ± 4.4	0.9	87 ± 1.5	1.1	9 ± 0.6	0.7	7 ± 1.5	0.9	43 ± 9.8	1.0
	100	33 ± 4.4	1.1	96 ± 14.6	1.2	14 ± 2.1	1.1	11 ± 2.3	1.5	43 ± 13.7	1.0
	316	27 ± 15.3	0.9	86 ± 4.6	1.1	9 ± 3.6	0.7	5 ± 3.6	0.7	41 ± 5.3	0.9
	1000	31 ± 5.6	1.0	101 ± 6.1	1.3	10 ± 2.5	0.7	11 ± 5.2	1.5	36 ± 10.0	0.8
	2500	32 ± 7.2	1.1	75 ± 4.9	0.9	16 ± 3.2	1.2	7 ± 8	1.0	46 ± 15.6	1.0
	5000	39 ± 11.5	1.3	80 ± 2.0	1.0	9 ± 2.1	0.7	8 ± 3.5	1.1	43 ± 7.4	1.0
2-AA*		1771 ± 208.7	58.4	1988 ± 240.2	24.5	113 ± 25-1	8.5	163 ± 2.9	22.3	120 ± 10.5	2.7
B[a]P*		-	-	298 ± 8.1	3.7	-	-	-	-	-	-

RC Revertant colonies: mean ± Standard deviation
MF Mutation factor: mean revertants (test item) / mean revertants (vehicle control)
* Positive control materials: 2-AA (2-Aminoanthracene), TA 98, TA 100, TA 1353, TA 1537 (2.5 µg/plate) and E. coli WP2 uvrA (10 µg/plate), B[a]p (Benzo[a]pyrene) TA 100 (5 µg/plate)

The number of revertants was only slightly affected by the test substance Bifenox. There was no significant increase of revertant colonies in comparison to the solvent control (MF = 1). Therefore, no mutagenic effects from the active

substance Bifenox were revealed up to 5000 µg/plate. The results from the pre-incubation test are demonstrated in **Table 3.8.1-3** and **Table 3.8.1-4.**

Table 3.8.1-3 Results from the pre-incubation test – without metabolic activation

Treat-ment	Dose [µg/plate]	TA 98		TA 100		TA 1535		TA 1537		E. coli WP2 uvrA	
		RC	MF	RC	MF	RC	MF	RC	MF	RC	MF
Aqua dest.		23 ± 2.0	1.5	84 ± 7.1	1.4	6 ± 1.5	0.8	9 ± 1.0	1.6	53 ± 1.5	1.5
DMSO		16 ± 1.2	1.0	61 ± 10.5	1.0	7 ± 2.6	1.0	6 ± 5.0	1.0	35 ± 3.0	1.0
Test Item	3.16	-	-	69 ± 9.5	1.1	-	-	-	-	-	-
	10.0	24 ± 3.2	1.5	63 ± 11.1	1.0	5 ± 2.5	0.7	5 ± 2.1	0.9	41 ± 3.1	1.2
	31.6	24 ± 5.0	1.5	75 ± 9.1	1.2	7 ± 1.7	1.0	5 ± 1.0	0.9	46 ± 10.4	1.3
	100	17 ± 2.3	1.1	57 ± 8.1	0.9	3 ± 2.0	0.4	4 ± 3.0	0.7	40 ± 3.6	1.1
	316	20 ± 4.0	1.3	63 ± 10.5	1.0	7 ± 2.6	1.0	4 ± 1.7	0.7	43 ± 9.6	1.2
	1000	18 ± 3.6	1.1	80 ± 16.8 B	1.3	10 ± 1.5	1.5	5 ± 2.1	0.8	45 ± 6.5	1.3
	2500	20 ± 2.5 P	1.3	65 ± 7.6 BP	1.1	6 ± 2.9 P	0.8	5 ± 4.0 P	0.9	41 ± 14.0 P	1.2
	5000	24 ± 8.4 P	1.3	78 ± 19.2 BPC	1.3	8 ± 2.5 P	1.1	9 ± 2.6 P	1.6	50 ± 16.9 P	1.4
4-NODP		335 ± 64.0	21.4	-	-	-	-	71 ± 14.6	12.6	-	-
NaN_3		-	-	241 ± 27.6	3.9	593 ± 68.0	84.7	-	-	-	-
MMS		-	-	-	-	-	-	-	-	658 ± 109.9	18.8

RC Revertant colonies: mean ± Standard deviation
MF Mutation factor: mean revertants (test item) / mean revertants (vehicle control)
* Positive control materials: NaN_3 (sodium azide), TA 100 and TA1535 (10 µg/plate), 4-NOPD (4-nitro-o-phenylene-diamine), TA 98 (10 µg/plate), TA 1537 (40 µg/plate), MMS (Methylmethanesulfonate), E. coli WP2 uvrA (1 µL)
B Background lawn reduced
P Precipitation
C Contamination

Table 3.8.1-4 Results from the pre-incubation test – with metabolic activation

Treat-ment	Dose [µg/plate]	TA 98		TA 100		TA 1535		TA 1537		E. coli WP2 uvrA	
		RC	MF	RC	MF	RC	MF	RC	MF	RC	MF
Aqua dest.		23 ± 3.8	0.9	91 ± 2.6	1.4	5 ± 2.0	1.0	9 ± 0.6	2.0	42 ± 4.6	0.9
DMSO		24 ± 4.4	1.0	63 ± 8.9	1.0	5 ± 0.0	1.0	4 ± 0.6	1.0	45 ± 5.1	1.0
Test Item	3.16	-	-	-	-	-	-	-	-	-	-
	10.0	23 ± 4.2	1.0	86 ± 4.9	1.4	7 ± 2.3	1.3	6 ± 1.5	1.5	76 ± 12.7	1.7
	31.6	28 ± 5.5	1.2	89 ± 15.1	1.4	5 ± 1.5	1.1	5 ± 1.5	1.1	72 ± 3.6	1.6
	100	24 ± 8.2	1.0	104 ± 11.1	1.7	10 ± 1.2	1.9	5 ± 2.1	1.5	85 ± 1.5	1.9
	316	21 ± 2.1	0.9	82 ± 9.0	1.3	7 ± 3.8	1.3	6 ± 3.1	0.8	82 ± 5.0	1.8
	1000	23 ± 4.0	1.0	73 ± 13.6	1.2	7 ± 4.6	1.3	4 ± 1.5	1.0	85 ± 5.5	1.9
	2500	22 ± 2.0	0.9	61 ± 13.8	1.0	8 ± 1.0	1.6	4 ± 1.2	1.5	52 ± 8.1	1.2
	5000	25 ± 8.1	0.9	94 ± 1.5	1.5	6 ± 4.0	1.2	6 ± 5.1	1.5	40 ± 11.9	0.9
2-AA*		1652 ± 47.3	68.8	1155 ± 338.1	18.3	44 ± 11	8.7	68 ± 5.0	15.8	124 ± 17.7	2.7
B[a]P*		-	-	247 ± 37.3	3.9	-	-	-	-	-	-

RC Revertant colonies: mean ± Standard deviation
MF Mutation factor: mean revertants (test item) / mean revertants (vehicle control)
* Positive control materials: 2-AA (2-Aminoanthracene), TA 98, TA 100, TA 1353, TA 1537 (2.5 µg/plate) and E. coli WP2 uvrA (10 µg/plate), B[a]p (Benzo[a]pyrene) TA 100 (5 µg/plate)

The number of revertants was only slightly affected by the test substance Bifenox. There was no significant increase of revertant colonies in comparison to the solvent control (MF = 1).

Precipitation was noted at 2500 and 5000 µg/plate in all experiments without S9 mix. In experiment II cytotoxic effects in TA 1535 (100 µg/plate, without S9 mix) were noted. Regarding results from higher concentrations of the test item that did not lead to a change, this effect is considered not to be biologically relevant. Therefore, no mutagenic effects from the active substance Bifenox were revealed up to 5000 µg/plate.

Both experiments showed the expected results for control plates. In all control assays that were performed under the same conditions, the positive controls show clear and very significant increases of the revertant colonies. In comparison, negative or solvent controls, did not reveal any effects.

Discussion and conclusion

The test item Bifenox technical was investigated for its potential to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and tester strain *E. coli* WP2 uvrA.

In two independent experiments several concentrations of the test item were used. Each assay was conducted with and without metabolic activation. The concentrations, including the controls, were tested in triplicate.

No toxic effects of the test item were noted in any of the five tester strains used up to the highest dose group evaluated with and without metabolic activation in experiment I and II, except in tester strain TA 100 in experiment II (without metabolic activation) toxic effects were noted at a concentration of 1000 µg/plate and higher. The reduction in the number of revertants down to a mutation factor of 0.4 found in experiment II in tester strain TA 1535 at a concentration of 100 µg/plate (without metabolic activation) was regarded as not biologically relevant due to lack of a dose-response relationship.

No biologically relevant increases in revertant colony numbers of any of the five tester strains were observed following treatment with Bifenox technical at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II. In tester strain E. coli WP2 uvrA in experiment I a mutation factor of 2.0 was observed at a concentration of 316 µg/plate (without metabolic activation). However, the corresponding revertant colony number was

within the range of the historical negative control data, no dose-response relationship was observed and the effect could not be reproduced in experiment II. Thus, the effect was considered as not biologically relevant.

The reference mutagens induced a distinct increase of revertant colonies indicating the validity of the experiment.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, Bifenox technical did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, Bifenox technical is considered to be non-mutagenic in this bacterial reverse mutation assay.

3.8.1.2 Study 2 – CA 5.4.1 *In vitro* genotoxicity/mutagenicity

Study reference

Report:	KCA 5.4.1/02, Anonymous (2016)
Title:	*In vitro* Mammalian Cell Gene Mutation Test (HPRT-Locus) in Chinese Hamster V79 Cells with Bifenox technical
Document No:	160131, 90018294
Guidelines:	OECD 476, (EC) No 440/2008 B 17, OPPTS 870.5300
GLP	Yes

Detailed study summary and results:

Test substance and procedure

The purpose of this study was to establish the potential of Bifenox (batch # D-20140741) to induce gene mutations in Chinese Hamster V79 cells by means of a HPRT-Locus *in vitro* assay.

The mammalian V79 were exposed to 0.25, 0.5, 1.0, 2.5, 5, 10, 25, 50, 100 and 250 µg/mL of Bifenox technical without metabolic activation and 0.5, 1.0, 2.5, 5, 10, 25, 50, 100 and 250 µg/mL with metabolic activation. Concentrations were selected due to cytotoxicity and solubility. Positive controls induced the appropriate response. There was no evidence of genotoxic effects from the test article Bifenox induced over background.

This study is classified as acceptable and it satisfies the requirement for Test Guideline 476 "*in vitro* Mammalian Cell Gene Mutation Tests using the HPRT and XPRT genes". Bifenox is concluded to be non-genotoxic, based on the results of this assay.

Results

In the pre-experiment for cytotoxicity, significant and dose dependent toxicity was seen without metabolic activation in dose groups receiving 50 µg/mL or higher doses. With metabolic activation, dose dependent and significant toxicity was observed in dose groups of 500 µg/mL or higher.

Therefore, concentrations for the main experiment were selected at 10 levels between 0.25 and 250 µg/mL without metabolic activation and 9 levels between 0.5 and 250 µg/mL with metabolic activation.

The main experiments were carried out without and with metabolic activation. The experiment with metabolic activation was performed by including liver microsomes and NADP for efficient detection of a wide variety of carcinogens requiring metabolic activation.

The selection of the concentrations used in the main experiment was based on data from the pre-experiments according to the OECD guideline 476. In the experiment 250 µg/mL was selected as the highest concentration (without and with metabolic activation). The results are demonstrated in **Table 3.8.1-5**.

Table 3.8.1-5 Results from the main experiment

Dose group	Concen-tration [µg/mL]	Without metabolic activation			With metabolic activation		
		Adjusted CE [%]	RS [%]	MF per 10^6 cells	Adjusted CE [%]	RS [%]	MF per 10^6 cells
NC1	0	111	89	35.0	104	97	31.9
NC2	0	105	84	35.8	104	97	28.9
S1	0	132	106	24.9	106	99	20.6
S2	0	118	94	29.7	108	101	34.6
1	0.25	113	91	45.7*	-	-	-
2	0.5	106	85	32.7	114	106	29.3
3	1.0	122	97	36.8	101	94	25.4
4	2.5	110	88	29.4	119	111	24.8
5	5.0	89	71	28.5	116	108	28.6
6	10	95	76	44.9*	122	114	22.0
7	25	96	77	44.3*	117	109	28.6
8	50	87	70	33.8*	105	98	38.9
9 P	100	87	70	47.1*	112	105	35.8
10 P	250	57	45	28.6	80	74	13.8**
EMS	300	130	104	241.2*	-	-	-
DMBA	1.5	-	-	-	113	106	248.4*

CE Cloning efficiency (adjusted for the number of colonies in non-selective medium to derive the mutant frequency)
RS Relative survival
MF Mutation factor
NC Negative control
S Solvent control (DMSO)
P Precipitation at the end of treatment
EMS Ethylmethanesulfonate [300 µg/mL]
DMBA 7,12-dimethylbenz(a)anthracene [1.5 µg/mL]
* Significant; ** significant decrease, therefore not relevant

At the end of the treatment precipitation of the test item was observed at concentrations of 100 µg/mL and higher (without metabolic activation) and at 250 µg/mL (with metabolic activation).

No growth inhibition (reduction of relative survival below 70%) was observed in the experiment with metabolic activation. Without metabolic activation growth inhibition was observed only at the highest concentration tested.

In the experiment without metabolic activation the relative survival was 45% for the highest concentration (250 µg/mL) evaluated. The highest concentration evaluated with metabolic activation was 250 µg/mL with a relative survival of 74%.

In the experiment without metabolic activation the mutant values of the negative controls, the solvent controls and most mutant values of the test item concentrations found were within the historical control data of the test facility about 9-36 mutants per 10^6 cells). The mutant frequencies of the negative controls were 35.0 and 35.8 mutants per 10^6 cells, of the solvent controls 24.9 and 29.7 mutants per 10^6 cells, and in the range of 28.5 to 47.1 mutants per 10^6 cells with the test item. The highest mutant frequency was observed at a concentration of 100 µg/mL (47.1 mutants per 10^6 cells) with a relative survival of 70%.

In the experiment with metabolic activation the mutant values of the negative controls, the solvent controls and all beside one mutant value of the test item concentrations found were within the historical control data of the test facility Eurofins Munich (about 8-37 mutants per 10^6 cells). The mutant frequencies of the negative controls were 31.9 and 28.9 mutants/10^6 cells, of the solvent controls 20.6 and 34.6 mutants per 10^6 cells, and in the range of 13.8 to 38.9 mutants per 10^6 cells with the test item.

The positive controls, DMBA (1.5 µg/mL) and EMS (300 µg/mL) showed statistically significant increases in mutant frequency, thereby demonstrating both the sensitivity and validity of the test systems.

Discussion and conclusion

In the experiment without and with metabolic activation all validity criteria were met. The mutant values of the negative controls fall within the historical data range of the test facility and the cloning efficiencies of the negative and solvent

controls are > 50%. The positive controls, DMBA and EMS showed statistically significant increases in mutant frequency, thereby demonstrating both the sensitivity and validity of the test systems.

A statistical analysis displayed that some of the mutant frequencies were significantly increased over those of the solvent controls. These frequencies are outside the historical data range of the test facility (9-36 mutants per 10^6 cells). Considering that the mutant frequency of the lowest concentration tested (0.25 µg/mL) was 45.7 mutants per 10^6 cells and thus in the same range as the mutant frequencies of the higher concentrations tested (10, 25, and 100 µg/mL) with 44.9, 44.3 and 47.1 mutants per 10^6 cells, respectively, this slight increase in mutant frequency and statistical significance is considered as not biologically relevant. Moreover, a dose-response relationship was not observed.

The highest mutant frequency was observed at a concentration of 50 µg/mL (38.92 mutants per 10^6 cells) with a relative survival of 98%. The mutant frequencies induced by the test item did not show a biologically relevant increase. None of the observed mutant frequencies were statistically significant increased over those of the solvent controls.

There was no cytotoxicity observed in the experiment with metabolic activation, only the highest dose in the experiment without metabolic activation showed reduced relative survival and was considered to be non-relevant.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, Bifenox technical did not cause gene mutations in the genome of V79 Chines Hamster cells. Therefore, Bifenox technical is considered to be non-mutagenic in this HPRT-Locus assay.

3.8.1.3 Study 3 – CA 5.4.1 *In vitro* genotoxicity/mutagenicity

Study reference

Report:	KCA 5.4.1/03, Anonymous (2016) amended 2017
Title:	*In vitro* Mammalian Chromosome Aberration Test in Chinese Hamster V79 cells with Bifenox technical
Document No:	160132, 90019295
Guidelines:	OECD 473, EC 440/2008 B. 10, EPA 712-C-98-223
GLP	Yes

The final report from June 2016 was amended in 2017 with additional data on Experiment II with metabolic activation for an exposure period of 4 hours. The August 2016 report is a full copy of the original report with inclusion of the new information.

Detailed study summary and results:

Test substance and procedure

In an *in vitro* mammalian chromosomal aberration assay, Chinese hamster V79 cells were exposed to Bifenox technical dissolved in DMSO at concentrations between 1 and 500 µg/mL in two independent experiments.

Bifenox technical was in two tested in two independent experiments up to cytotoxic and insoluble concentrations during exposure periods of 4 h with (5, 10, 25, 50, 100, 250, 350 and 500 µg/mL) and without metabolic activation (5, 10, 25, 50, 100 and 250 µg/mL) and in the second experiment 21 hours without metabolic activation (1, 2, 5, 10, 25, 50 and 100 µg/mL) and 4 h with metabolic activation (20, 40, 75, 125, 200, 325, 450 and 600 µg/mL).

The positive controls induced the appropriate response. There was no evidence of induced chromosome aberration from Bifenox technical when compared to historical control data from the institution.

This study is classified as acceptable. This study satisfies the requirements for Test Guideline OECD 476, EC 440/2008 B. 10 and EPA 712-C-98-223 for *in vitro* mammalian chromosome aberration.

MATERIALS AND METHODS

MATERIALS

Test Material	Bifenox technical
Description:	Yellow solid
Batch #:	D-20140741
Purity:	98.0%
Expiry date:	June 2017
Storage conditions:	Room temperature
Solvent used:	Dimethylsulfoxide (DMSO)
Positive control Materials	
Without metabolic activation:	Ethylmethanesulfonate (EMS) Batch # BCBQ0451V, Sigma
With metabolic activation:	Cyclophosphamide (CPA) Batch # SLBG4216, Sigma
Test system	Chinese Hamster V79 cells (ATCC, CCL-93) from Eurofins Munich, stored over liquid Nitrogen
Complete culture medium	MEM medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL penicillin/streptomycin solution, 2 mM L-glutamine, 2.5 µg/mL amphotericin, 25 mM HEPES
Treatment medium	Complete culture medium without FBS
Test concentrations	Experiment I (4 hours): without metabolic activation: 5, 10, 25, 50, 100 and 250 µg/mL, with metabolic activation: 5, 10, 25, 50, 100, 250, 350 and 500 µg/mL, Experiment II (21 hours): without metabolic activation: 1, 2, 5, 10, 25, 50 and 100 µg/mL Experiment II (4 hours): with metabolic activation: 20, 40, 75, 125, 200, 325, 450 and 600 µg/mL

TEST PERFORMANCE

In-life dates

18. February 2016 – 25. June 2016 at Eurofins BioPharma, Planegg, Germany.

Pre-Experiment for Toxicity

A pre-experiment was conducted under identical conditions as described for the main experiment. The following concentrations were tested without and with S9 mix: 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 1500 and 2000 µg/mL.

Exposure Concentrations

On the basis of the data and the observations from the pre-experiment the following concentrations were selected for the main experiments I and II

Table 3.8.1-6 Experimental design

S9 mix	Exposure interval	Preparation interval	Concentrations in µg/mL							
Experiment I										
-	4 h	21 h	5	10	25	50 P	100 P	250 P	-	-
+	4 h	21 h	5	10	25	50	100 P	250 P	350 P	500 P
Experiment II										
-	21 h	21 h	1	2	5	10	25	50	100 P	-
+	4 h	21 h	20	40	75 P	125 P	200 P	325 P	450 P	600 P

P Precipitation was observed at the end of treatment

Experimental performance

Experiment I

Exposure time 4 h (without and with S9 mix): Two days after seeding of the cells, the culture medium was replaced with serum-free medium containing the different concentrations of the test item and S9 mix (only with metabolic activation). Additional negative and positive controls were performed without and with metabolic activation. 4 h after the treatment the cultures were washed twice with PBS and then the cells were cultured in complete culture medium for 16 ± 2 hours until preparation of the cells (21 h after short term treatment).

Experiment II

Exposure time 21 h (without S9 mix) and 4 h (with S9 mix): For the 21 h treatment time, two days after seeding of the cells the culture medium is replaced with complete medium containing the different concentrations of the test item. This medium is not changed until preparation of the cells. All cultures were incubated at 37 ± 1 °C in a humidified atmosphere with 5.0% CO_2 (95.0% air). In the 4 h experiment, S9 mix was added to the medium and 4 h after the treatment, the cultures were washed twice with PBS and then the cells were cultured in complete culture medium for 16 ± 2 hours until preparation of the cells (21 h after short term treatment).

Analysis of Metaphase Cells

Evaluation of the cultures was performed according to a standard protocol using microscopes with 100 × oil immersion objectives. If observed, structural chromosomal aberrations, including breaks, fragments, deletions, exchanges and chromosomal disintegration were recorded. Gaps were recorded as well but not included in the calculation of the aberration rates. The remaining visible chromosome regions should not be dislocated either longitudinally or laterally. At least 300 well spread metaphases per concentration and validity controls were scored for cytogenetic damage.

To describe a cytotoxic effect the cell count (RICC) was determined. Additionally the number of polyploid cells is scored. Polyploid means a near tetraploid karyotype in the case of this aneuploid cell line.

Evaluation of Results

Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, the increase is dose-related when evaluated with an appropriate trend test and if any of the results are outside the distribution of the historical negative control data.

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, there is no concentration-related increase when evaluated with an appropriate trend test and if all results are inside the distribution of the historical negative control data.

RESULTS

PRE-EXPERIMENT FOR TOXICITY

According to the guidelines the highest recommended concentration was 2000 µg/mL. Precipitation of the test item was noted from concentrations of 125 µg/mL without metabolic activation and 250 µg/mL with metabolic activation. The relative increase in cell count (RICC) was used as parameter for toxicity.

The results from the pre-experiment without metabolic activation are summarized in **Table 3.8.1-7**, with metabolic activation in **Table 3.8.1-8**.

Table 3.8.1-7 Results from the pre-experiment without metabolic activation

Dose group	Concentration µg/mL	Cell count/mL × 10^3	RICC [%]
C	0	158	104
S	0	153	100
1	7.8	119	76
2	15.6	132	85
3	31.3	100	63
4	62.5	89	55
5 [P]	125	66	39
6 [P]	250	66	39
7 [P]	500	45	25

RICC Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the solvent control groups. The cell count was determined by a cell counter per culture for each test group 21 h after the beginning of the treatment. 5 × 10^4 cells were seeded in the cell culture flasks.
C Negative Control (Culture Medium)
S Solvent Control DMSO
P Precipitation determined microscopically after test item treatment

Table 3.8.1-8 Results from the pre-experiment with metabolic activation

Dose group	Concentration [µg/mL]	Cell count/mL × 10^3	RICC [%]
C	0	152	88
S	0	172	100
1	7.8	157	91
2	15.6	153	88
3	31.3	161	94
4	62.5	164	95
5 [P]	125	169	99
6 [P]	250	153	88
7 [P]	500	75	40
8 [P]	1000	70	37
9 [P]	1500	62	32
10 [P]	2000	67	36

RICC Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the solvent control groups. The cell count was determined by a cell counter per culture for each test group 21 h after the beginning of the treatment. 5 × 104 cells were seeded in the cell culture flasks.
C Negative Control (Culture Medium)
S Solvent Control DMSO
P Precipitation determined microscopically after test item treatment

EXPERIMENT I

The concentrations evaluated in the main experiment based on the results obtained in the pre-experiment (**Table 3.8.1-8**).

Toxicity

In experiment I without metabolic activation, a biologically relevant decrease of the RICC (decrease below 70% RICC) was noted at 25 µg/mL and higher (65% at 25 µg/mL, 44% at 50 µg/mL, 30% at 100 µg/mL and 23% at 250 µg/mL. With metabolic activation a biologically relevant decrease of the RICC was noted at 250 µg/mL and higher (68% at 250 µg/mL, 37% at 350 µg/mL and 39% at 500 µg/mL). The results are summarized in **Table 3.8.1-6**.

Table 3.8.1-9 Experiment I – summary of cytotoxicity data

Dose group	Concentration [µg/mL]	Polypoid cells mean	Cell count/mL × 10^3 mean	RICC [%]
Without metabolic activation				
C	0	0	105	107
S	0	0.5	99	100
1	8	n.d.	92	92
2	16	0.5	81	80
3	31	0	68	65
4 [P]	63	0	49	44
5 [P]	125	n.d.	37	30
6 [P]	250	n.d.	30	23
EMS	600	0.5	89	88
With metabolic activation				
C	0	0	95	88
S	0	0	107	100
1	8	n.d.	97	89
2	16	n.d.	79	71
3	31	0	100	93
4	63	0	98	91
5 [P]	125	0	96	89
6 [P]	250	n.d.	76	68
7 [P]	350	n.d.	46	37
8 [P]	500	n.d.	48	39
CPA	1	0	80	72

The number of polyploid cells was determined in 150 cells per culture of each test group.

RICC Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the solvent control groups. The cell count was determined by a cell counter per culture for each test group 21 h after the beginning of the treatment. 5×10^4 cells were seeded in the cell culture flasks.

C Negative Control (Culture Medium)

S Solvent Control (DMSO)

EM Positive Control (without metabolic activation: Ethylmethanesulfonate)

CPA Positive Control (with metabolic activation: Cyclophosphamide)

P Precipitation determined microscopically after test item treatment

n.d. not determined

Clastogenicity

In experiment I without metabolic activation the aberration rate of the negative control (1.0%), solvent control (2.7%) and the test item concentrations 10 µg/mL (2.3%), 25 µg/mL (2.0%) and 50 µg/mL (1.3%) were within the historical control data of the testing facility (0.0% – 4.0%). In addition, no dose-response relationship was observed. The number of aberrant cells found in the concentrations treated with the test item did not show a statistically significant increase compared to the corresponding solvent control.

In experiment I with metabolic activation, the aberration rates of the negative control (2.0%), solvent control (3.0%) and all concentrations treated with the test item (25 µg/mL - 2.3%, 50 µg/mL – 3.7% and 100 µg/mL - 2.0%) were within the historical control data of the testing facility (0.0% – 4.3%). In addition, no dose-response relationship was observed. The

number of aberrant cells found in the concentrations treated with the test item did not show a statistically significant increase compared to the corresponding solvent control.

The aberration rates from experiment I with and without metabolic activation are summarized in **Table 3.8.1-10**.

Table 3.8.1-10 Experiment I – summary of aberration rates

Dose group	Concentration [µg/mL]	Treatment time	Fixation interval	Mean % aberrant cells	
				Incl. gaps	Excl. gaps
Without metabolic activation					
C	0	4 h	21 h	1.3	1.0
S	0	4 h	21 h	2.7	2.7
2	10	4 h	21 h	5.3	2.3
3	25	4 h	21 h	3.7	2.9
4 P	50	4 h	21 h	2.7	1.3
EMS	600	4 h	21 h	12.3	9.7
With metabolic activation					
C	0	4 h	21 h	4.0	2.0
S	0	4 h	21 h	4.7	3.0
3	25	4 h	21 h	4.3	2.3
4	50	4 h	21 h	6.7	3.7
5 P	100	4 h	21 h	2.7	2.0
CPA	1.11	4 h	21 h	14.4	12.0

300 cells evaluated for each concentration, except for the positive control CPA (250 cells).
C Negative Control (Culture Medium)
S Solvent Control (DMSO)
EMS Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA Positive Control (with metabolic activation: Cyclophosphamide)
P Precipitation determined microscopically after test item treatment

EXPERIMENT II

Toxicity

In experiment II without metabolic activation, a biologically relevant decrease of the RICC was detected at 25 µg/mL (67%), 50 µg/mL (63%) and 100 µg/mL (57%). With metabolic activation a biologically relevant decrease of the RICC was noted at 325 µg/mL and higher (60% at 325 µg/mL, 37% at 450 µg/mL and 10% at 600 µg/mL). The results are summarized in **Table 3.8.1-11**.

Table 3.8.1-11 Experiment II – Summary of cytotoxicity data

Dose group	Concentration [µg/mL]	Polypoid cells Mean	Cell count/mL × 10^3 Mean	RICC [%]
Without metabolic activation (21 h)				
C	0	0	152	116
S	0	0.5	133	100
1	1	n.d.	150	114
2	2	n.d.	143	108
3	5	0	109	81
4	10	n.d.	114	85
5	25	1	92	67
6	50	0	88	63
7 [P]	100	0	79	57
EMS	600	0	108	80
With metabolic activation (4 h)				
C	0	0.5	90	99
S	0	1.0	91	100
1	20	0.5	95	105
2	40	1.0	91	100
3 [P]	75	0.0	100	110
4 [P]	125	n.d.	103	115
5 [P]	200	n.d.	93	102
6 [P]	325	n.d.	59	60
7 [P]	450	n.d.	40	37
8 [P]	600	n.d.	18	10
EMS	1.11	1.0	70	73

The number of polyploid cells was determined in 150 cells per culture of each test group.
RICC Relative Increase in Cell Count, calculated by the increase in cell number of the test groups compared to the solvent control groups. The cell count was determined by a cell counter per culture for each test group 21 h after the beginning of the treatment. 5×10^4 cells were seeded in the cell culture flasks.
C Negative Control (Culture Medium)
S Solvent Control (DMSO)
EMS Positive Control (without metabolic activation: Ethylmethanesulfonate)
P Precipitation determined microscopically after test item treatment
n.d. not determined

Clastogenicity

In experiment II without metabolic activation the aberration rate of the negative control (2.0%), solvent control (1.0%) and the test item concentrations 5 µg/mL (0.7%), 25 µg/mL (1.6%), 50 µg/mL (1.3%) and 100 µg/mL (1.3%) were within the historical control data of the testing facility (0.0% – 4.0%). In addition, no dose-response relationship was observed. The number of aberrant cells found in the concentrations treated with the test item did not show a statistically significant increase compared to the corresponding solvent control.

In experiment II with metabolic activation, the aberration rate of the negative control (1.0%), solvent control (1.3%) and the test item concentrations 20 µg/mL (0.0%), 40 µg/mL (1.3%) and 75 µg/mL (1.7%) were within the historical control data of the testing facility (0.0% – 4.0%). In addition, no dose-response relationship was observed. The number of aberrant cells found in the concentrations treated with the test item did not show a statistically significant increase compared to the corresponding solvent control.

The aberration rates from experiment II without metabolic activation are summarized in **Table 3.8.1-12.**

Table 3.8.1-12 Experiment II – Summary of Aberration Rates

Dose group	Concentration [µg/mL]	Treatment Time	Fixation Interval	Mean % aberrant cells	
				Incl. Gaps	Excl. Gaps
Without metabolic activation					
C	0	21 h	21 h	3.0	2.0
S	0	21 h	21 h	1.7	1.0
3	5	21 h	21 h	1.7	0.7
5	25	21 h	21 h	3.1	1.6
6	50	21 h	21 h	2.3	1.3
7[P]	100	21 h	21 h	2.7	1.3
EMS	600	21 h	21 h	24.0	21.6
With metabolic activation (4 h)					
C	0	4 h	21 h	1.7	1.0
S	0	4 h	21 h	1.7	1.3
1	20	4 h	21 h	0.7	0.0
2	40	4 h	21 h	2.0	1.3
3 [P]	75	4 h	21 h	3.3	1.7
CPA	1.11	4 h	21 h	31.3	26.1

300 cells evaluated for each concentration, except for the positive control CPA (250 cells).
C Negative Control (Culture Medium)
S Solvent Control (DMSO)
EMS Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA Positive Control (with metabolic activation: Cyclophosphamide)
P Precipitation determined microscopically after test item treatment

Discussion and conclusion

In an *in vitro* chromosome aberration assay, the test item Bifenox technical was investigated for the potential to induce structural chromosomal aberrations in Chinese hamster V79 cells in the absence and presence of metabolic activation. The chromosomal aberration assay is considered acceptable as the number of aberration found in the negative and solvent controls were within the range of historical laboratory control data. Furthermore, the positive controls induced responses that are compatible with those generated in the historical positive control data base and produced a statistically significant increase compared with the concurrent solvent control.

Precipitation

In experiment I, precipitation of the test item was noted without metabolic activation at a concentration of 50 µg/mL and with metabolic activation at a concentration of 100 µg/mL by microscopic evaluation. In experiment II, precipitation of the test item was seen without metabolic activation at a concentration of 100 µg/mL.

Toxicity

In experiment I without metabolic activation, a biologically relevant decrease of the RICC (decrease below 70% RICC) was noted at 25 µg/mL and higher (65% at 25 µg/mL, 44% at 50 µg/mL, 30% at 100 µg/mL and 23% at 250 µg/mL). With metabolic activation a biologically relevant decrease of the RICC was noted at 250 µg/mL and higher (68% at 250 µg/mL, 37% at 350 µg/mL and 39% at 500 µg/mL). In experiment II without metabolic activation, a biologically relevant decrease of the RICC was detected at 25 µg/mL (67%) 50 µg/mL (63%) and 100 µg/mL (57%) and with metabolic activation a decrease of the RICC was noted at 325 µg/mL and higher (60% at 325 µg/mL, 37% at 450 µg/mL and 10% at 600 µg/mL).

Clastogenicity

In all experiments with and without metabolic activation, the aberration rates of the negative controls, solvent controls and the test item concentrations were within the historical control data of the testing facility. In addition, no dose-response

relationship was observed. The number of aberrant cells found in the concentrations treated with the test item did not show a statistically significant increase compared to the corresponding solvent control.

EMS and CPA were used as positive controls and induced distinct and biologically relevant increases in cells with structural chromosomal aberrations, thus proving the ability of the test system to indicate potential clastogenic effects. No statistically significant increase of cells with chromosomal aberrations was noted in the dose groups of the test item evaluated in experiment I and II without and with metabolic activation. Also, no statistically significant increase was observed in experiment I without and with metabolic activation and in experiment II without metabolic activation. No biologically relevant increase in the frequencies of polyploid cells was found after treatment with the test item.

Conclusion

In conclusion, it can be stated that during the described *in vitro* chromosome aberration test and under the experimental conditions reported, the test item Bifenox technical did not induce structural chromosomal aberrations in the V79 Chinese hamster cell line.

Therefore, the test item Bifenox technical is considered to be non-clastogenic in this chromosome aberration test.

3.8.1.4 CA 5.2.6 – further *in vitro* studies

Studies on the potential of Bifenox to cause mutagenic effects that have already been submitted in the context of the inclusion of Bifenox in Annex I of Council Directive 91/414/EEC were re-evaluated for the confirmation of the non-genotoxic potential. There was no evidence, that Bifenox causes genetic changes in bacterial or mammalian cells.

Table 3.8.1-13 Results of *in vitro* genotoxicity assays with Bifenox

Table 3.8.1-13 Results of *in vitro* genotoxicity assays with BifenoxTest	**Test system**	**Conditions**	**Test substance**	**GLP / Guidelines**	**Result**	**Klimisch category**	**Reference**
Bacterial mutagenicity	*S. typhimurium* (strain TA100, TA1535, TA98, TA1537, TA1538 and TA102)	Plate incorporation assay and a preincubation method at 3.16 to 316 µg/plate	Bifenox Batch # 10830 Purity: 99.1%	GLP / OECD 471	Negative	1*	Anonymous, 2005a (DAR 2006)
Bacterial mutagenicity	*S. typhimurium* (TA100, TA1535, TA98, TA1537 and TA1538), *E. coli* WP2 uvrA	Plate incorporation assay, with and without S9 mix 10-5000 µg/plate	Bifenox Batch # not stated Purity: 99.5%	No GLP statement / assay was conducted according to standard procedures (Ames et al, 1975) on which OECD 471 is based / test is judged to be valid	Negative	2**	Anonymous, 1982 (DAR 2006)
Bacterial mutagenicity	*S. typhimurium* (TA100, TA1535, TA98, TA1537 and TA1538)	Plate incorporation assay, with and without S9 mix 10000, 5000, 2500, 500 or 100 µg/plate	Bifenox Batch # MCTR-12-79 (MRI #248) Purity not stated	No GLP statement / plate incorporation assay was conducted according to standard procedures (Ames et al, 1975) on which OECD 471 is based / test is judged to be valid	Negative	2**	Anonymous, 1979 (DAR 2006)
Chromosomal aberration	CHO cells	25 - 2510 µg/mL	Bifenox Lot # 3123142024 Purity: 97%	No GLP statement / assay was conducted according to standard procedures on which OECD 473 is based / test is judged to be valid	Negative	1*	Anonymous, 1985 (DAR 2006)

Table 3.8.1-13 Results of *in vitro* genotoxicity assays with BifenoxTest	**Test system**	**Conditions**	**Test substance**	**GLP / Guidelines**	**Result**	**Klimisch category**	**Reference**
Mammalian cell gene mutation	Mouse lymphoma L5178Y cells ($tk^{+/-}$ system)	19.53 to 312.5 µg/mL with and w/o S9 mix; in the second experiment w/o S9 mix concentrations ranging from 9.77 to 156.25 µg/mL were used.	Bifenox Batch # 10830 Purity: 99.1%	GLP / OECD 476	Negative	1*	Anonymous, 2005b (DAR 2006)
Mammalian cell gene mutation	Mouse lymphoma L5178Y cells ($tk^{+/-}$ system)	w/o S9 mix: 133-1000 µg/mL, with S9 mix: 18 - 133 µg/mL	Bifenox Batch # MCTR-12-79 (MRI #248) Purity not stated	No GLP statement / assay was conducted according to standard procedures on which OECD 476 is based / test is judged to be valid	Negative	2**	Anonymous, 1979 (DAR 2006)
Mammalian cell gene mutation	CHO-cells (HGPRT system)	50 - 500 µg/mL with S9 mix, 30 – 250 µg/mL without S9 mix	Bifenox Batch # and purity not stated	GLP / assay was conducted according to standard procedures on which OECD 476 is based / test is judged to be valid	Negative	2**	Anonymous, 1983 (DAR 2006)
UDS assay	Rat hepatocytes	8 doses from 100 µg/mL to 0.5 µg/mL	Bifenox Lot # 16230 Purity not stated	GLP / assay was conducted according to standard procedures on which OECD 482 is based / test is judged to be valid	Negative	1*	Anonymous, 1981 (DAR 2006)

* Category 1: Reliable without restrictions - useful, check relevance for intended purpose
** Category 2: Reliable with restrictions - potentially useful, check relevance for intended purpose

Bifenox, mutagenicity study using *S. typhimurium*, reverse mutation assay (Anonymous, 2005a)

(From April 2017 RAR)

GLP status: yes

Guideline: study is conforming to dir 2000/32/EEC Annex 4D, or dir 92/69/EEC method B 14, dir 84/449 or OECD 471(1997-83) guideline.

Material and methods: *Salmonella typhimurium* strains TA 100, TA1535, TA98, TA 1537, TA1538, and TA 102 were exposed to bifenox (Agan, batch n°10830, 99.1%) dissolved in DMSO using the plate incorporation assay and a preincubation method. Five concentrations (316, 100, 31.6, 10, 3.16 µg/plate) were tested with and without S9 mix (from rat liver pretreated with Arochlor 1254). Positive controls (cyclophosphamide, sodium azide, 9-aminoacridine, 2-nitrofluorene, 2-aminoanthracene) gave the expected results.

The study is acceptable.

Findings: bifenox was examined in a preliminary cytotoxicity test in strain TA 100. Ten concentrations ranging from 0.316 to 5000 µg/plate were tested. A precipitation was noted from 316 µg/plate onwards.

In the main study, five concentrations ranging from 3.16 to 316 µg/plate were tested in the plate incorporation assay and in the preincubation test with and without metabolic activation system. No mutagenic effect was observed in the two independent tests.

Conclusion: bifenox did not induce gene mutations towards *S.typhimurium* under these experimental conditions.

Bifenox- mutagenicity study using bacterial strains (Anonymous, 1982)

(From April 2017 RAR)

GLP status: no

Guideline: study is not fully conforming to dir 2000/32/EEC Annex 4D, or dir 92/69/EEC method B 14, dir 84/449 or OECD 471(1997-83) guideline.

Deviation from official protocol: phenotypic characteristics of strains were not checked. The study was not repeated. Plates were duplicated.

Absence of negative control. Preparation of S9 not reported.

Material and methods: *Salmonella typhimurium* strains TA 100, TA1535, TA98, TA 1537, TA1538, and E.coli WP2 uvrA were exposed to bifenox (batch n°? 99.5%) dissolved in DMSO using the plate incorporation assay. Five concentrations (5000; 1000, 500, 100, 50 or 10 µg/plate) were tested with and without S9 mix. Positive controls (2 AF, sodium azide, 9-aminoacridine, 2-nitrofluorene, 2-aminoanthracene) gave the expected results.

The study is acceptable.

Findings: Negative results were observed with or without metabolic activation system under these experimental conditions.

Conclusion: bifenox did not increase mutations rates in the different strains of Salmonella *thyphimurium*.

***Salmonella*/mammalian-microsome plate incorporation mutagenesis assay (Anonymous, 1979)**

(From April 2017 RAR)

GLP status: no, only attest of QAU.

Guideline: study is not fully conforming to dir 2000/32/EEC Annex 4D, or dir 92/69/EEC method B 14, dir 84/449 or OECD 471(1997-83) guideline.

Deviation from official protocol: The study was not repeated. Standard deviations not reported. TA 102 and *E.coli* not tested.

Material and methods: *Salmonella typhimurium* strains TA 100, TA1535, TA98, TA 1537 and TA1538 were exposed to bifenox (batch n°. 16230, 98.3%) dissolved in DMSO using the plate incorporation assay with and without metabolic activation of rat liver S9 from Arochlor 1254 treated rats. 8 concentrations (3, 9, 30, 95, 305, 911, 3125, 10000 µg/plate)

were tested for toxicity towards strain TA 100. The test was performed at 100, 500, 2500, 5000 or 10000 µg/plate. Positive controls (9-AA, propane sultone and 2-AA) gave the expected results.

The study is acceptable

Findings: A preliminary cytotoxicity test was performed using strain TA 100 at concentrations from 3 up to 10000 µg/plate. No cytotoxic effects were seen. Bifenox did not induce mutations in the different *S.typhimurium* strains with or without S9 mix.

Conclusion : Bifenox did not increase the reversion rate in the different *S.typhimurium* strains under these experimental conditions.

***In vitro* chromosomal aberration assay on bifenox technical, CHO cells (Anonymous,1985)**

(From April 2017 RAR)

GLP status: attest of QAU

Guideline: study is not fully conforming to dir 2000/32/EEC, Annex 4A or dir 92/69/EEC or dir 84/449/EEC, or OECD guideline 473(1997-1983).

Deviation from official protocol: the study was not repeated (this is conform to dir 84/449). Expression of results: % abnormal cells excluding gaps only reported (this is conform for OECD guideline 1983); 15 metaphases/culture examined for positive control. Too short exposure time with S9 (2 h). Harvest at 8/10 h and 18/19 h: OK for dir 84/449 and 92/69.

Material and methods: Chinese hamster ovary cells were exposed to bifenox (97 %, n°3123142024) dissolved in DMSO with or w/o metabolic activation system (S9 mix) from Arochlor 1254 pretreated rats. Concentrations tested ranging from 25-2510 µg/ml. Without S9, single cell flasks were exposed to test compound (8 hr exposure + initial overnight exposure) or repeat overnight exposure. Two harvest time were used, 8 hour and 18h (overnight).mWith S9: exposure time was 2 hr and growth period of 8 hr or overnight (17 hr). 500 cells were scored (100 metaphases). No repeat experiment performed. Criteria for positive response well defined. Mitomycin and cyclophosphamide were used as positive control. The study is acceptable.

Findings: Bifenox was tested at 25, 75, 250 and 750 µg/ml without S9mix for 8 hr. None of the analyzed concentrations induced aberration frequencies different from the negative control. Mitomycin induced a significant increase in aberration frequency. After 18 hr exposure, negative results were seen.

With S9mix, bifenox was tested at 125, 250, 400, 1260 and 2510 µg/ml for 2 hr + 8 hr (growth period). Very low mitotic index was reported at 1260 µg/ml. No chromosomal aberrations were observed. When the growth period was 17 hr, mitotic indexes at concentrations > 400 µg/ml were extremely low. No chromosomal aberrations were observed.

Cyclophosphamide induced a significant increase in aberration frequencies.

Conclusion: Bifenox was assayed for its ability to induce chromosomal aberrations in CHO cells in the presence and absence of metabolic activation system. **Chromosomal aberrations were not observed under the conditions of the assay employed.**

Mutagenicity study of bifenox in the mouse lymphoma test (Anonymous, 2005b)

(From April 2017 RAR)

GLP status: yes

Guideline: study is conforming to dir 2000/32/EEC, Annex 4E or dir 87/302/EEC or OECD 476(1997-1984) guideline.

Material and methods: L5178Y TK+/- cells were exposed to bifenox (Agan, batch 10830 purity: 99.1%) dissolved in DMSO was tested from 19.53 up to 312.5 µg/ml without metabolic activation system, 3 hours incubation and in two

experiments with metabolic activation system. In the second experiment w/o S9 mix (24 hour incubation) concentrations ranging from 9.77 up to 156.25 µg/ml were used. This experiment with S9mix was carried out twice. An initial toxicity test was performed at 25 up to 5000 µg/ml for 3 hours. MMS and 3-MC were used as positive controls. S9 was from liver of Arochlor 1254 pretreated rats. Criteria of acceptability of the test are well defined. Mutant frequencies induced by positive controls were appropriate. The study is acceptable.

Findings: cytotoxicity and compound precipitation was noted in the preliminary experiment from concentrations of 250 µg/ml onwards in the experiments w or w/o metabolic activation system.

In the main test, cytotoxicity was noted at the top dose of 312 or 156.25 µg/ml in the first and 2nd experiment w/o metabolic activation system (3 or 24 hr), respectively, immediately after treatment and in the following plating for 5-trifluoro-thymidine resistance. In the experiment with metabolic activation, cytotoxicity was noted at the top dose of 312.5 µg/ml, in the 1st and 2nd experiment, immediately after treatment and in the following plating for 5-trifluoro-thymidine resistance. Positive controls and solvents gave the expected results.

The mutation frequencies of cultures treated with bifenox ranged from 18.29 to 34.41/106 clonable cells in the experiments w or w/o metabolic activation. These results were in the range of the solvent controls and hence, no mutagenicity was observed according to the criteria for assay evaluation. No change was observed in the ratio of small to large mutant colonies, ranging from 0.0 to 2.0 for bifenox treated cells and from 1.02 to 2.03 for the solvent controls.

Conclusion: under the present test conditions, bifenox, tested up to cytotoxic concentrations with or w/o S9 mix was negative with respect to the mutant frequency in the LK5178Y TK+/- mammalian cell mutagenicity test.

Evaluation of compound MCRT-12-79 (MRI 248) for mutagenic potential employing the L5178Y TK+/- mutagenesis assay (Anonymous, 1979)

(From April 2017 RAR)

GLP status: no

Guideline: study is not fully conforming to dir 2000/32/EEC, Annex 4E or dir 87/302/EEC or OECD 476(1997-1984) guideline.

Deviation from official protocol: Absence of mycoplasma not verified. Colony sizing was not performed. Study was not repeated.

Material and methods: L5178Y TK+/- cells were exposed to bifenox (technical, batch? purity?) dissolved in acetone was tested from 1000-to 13 µg/ml. Exposure time: 4 hr with or w/o S9 mix. After 3-day expression, 7 cultures w/o S9 and 8 cultures with S9 mix were selected for cloning based on their degree of toxicity. W/o S9, cultures were cloned at 1000, 750, 563, 316, 237, 178 and 133 µg/ml. The cultures receiving metabolic activation were cloned at 133, 100, 75, 56, 42, 32, 24, and 18 µg/ml. An initial toxicity test was performed at 0.05 up to 5000 µg/ml. EMS and 7,12 DMBA were used as positive controls. S9 was from liver of Arochlor 1254 pretreated rats. Criteria of acceptability of the test are well defined. Mutant frequencies induced by positive controls were appropriate. The study is acceptable.

Findings: Inhibition of suspension growth was seen at 50, 500 and 5000 µg/ml with or w/o metabolic activation system. The cultures treated w/o activation were cloned over a range of concentrations, which produced from 24% to 66% suspension growth, and the cultures receiving S9 metabolic activation system were cloned over a range of concentrations that produced from 0% to 123% suspension growth. The results of the assay indicate that bifenox both with and without metabolic activation is negative in the mouse lymphoma assay.

Conclusion: Bifenox did not induce mutation in the TK locus of L5178Y TK+/- cells when tested in the presence and absence of metabolic activation system.

CHO/HGPRT mammalian cell forward gene mutation assay with bifenox technical (Anonymous, 1983)

(From April 2017 RAR)

GLP status: no, only attest of QAU

Guideline: study is not fully conforming to dir 2000/32/EEC, Annex 4A or dir 87/302 or OECD guideline 476(1997-1984).

Deviation from official protocol: the study was not repeated but all treatment groups were tested in duplicate. Absence of mycoplasm not verified. Origin of S9 is not given.

Material and methods: Chinese hamster ovary cells K1-BH4 were exposed to bifenox (purity? technical bifenox) dissolved in DMSO with or w/o metabolic activation system (S9 mix). A preliminary cytotoxicity test was performed at 0.03, 0.1, 0.33, 1, 3.33, 10, 33.33, 100, 333, 1000 µg/ml medium. Selected doses for the test were 30, 50, 100 and 200 µg/ml w/o S9mix and 50, 100, 300,and 400 µg/ml with activation system. Ethylmethanesulfonate and dimethylnitrosamine were used as positive control. The compound precipitated at 100, 200, 250, 300, 400, and 500 µg/ml. there was no apparent change in the physical state of the compound. The study is acceptable.

Findings: In the preliminary cytotoxicity test, complete cytotoxicity was seen at 333 and 1000 µg/ml w/o S9mix and at 1000 µg/ml with S9mix. At 100 µg/ml, w/o S9mix, relative survival was of 84% and at 333 µg/ml with S9mix of 63%. The high dose chosen for use in the CHO/HGPRT test was 250 µg/ml and 500 µg/ml with or without S9mix, respectively. There were no observed increases in mutation frequencies in the CHO cell line treated with bifenox.

Conclusion: The results for bifenox were negative in the CHO/HGPRT mammalian cell forward gene mutation test with or without metabolic activation system in these experimental conditions.

Evaluation of bifenox technical in the primary hepatocyte unscheduled DNA synthesis assay (Anonymous, 1981)

(From April 2017 RAR)

GLP status: yes, no attest of competent authority.

Guideline: study is not fully conforming to dir 87/302/EEC, Annex V B or OECD guideline 482 (1986).

Deviation from official protocol: experiment was not repeated.

Material and methods: primary rat hepatocyte (adult male Fischer 344 rats) cultures were used. Bifenox (lot 16230, 98.3%) was solubilized in DMSO. The hepatocytes were collected at approximately 81% viability, and the 18-hour treatment period with bifenox was initiated about 3.5 hours after a 1.5-hour settling period. The surviving population at the time of treatment was estimated to be about 73% of the number of viable seeded cells, and the culture was 99% viable. Over the course of the next 20-23 hr (including the treatment period), the solvent control viable cell count remained at 101% of the count at initiation of treatment. Complete solubility was achieved for final concentrations of 10 µg/ml and lower. Cloudiness appeared at 25 µg/ml and clumpy precipitate was obtained at 1000 µg/ml. 15 concentrations ranging from 1000 µg/ml to 0.025 µg/ml were applied to the cells. At 250 µg/ml and higher, excessive toxicity was seen. Therefore, 8 doses from 100 µg/ml to 0.5 µg/ml were selected. This range exceeded the solubility limit and included treatments causing toxicities similar to and greater than those necessary for 2AAF to induce large increases in nuclear labeling. The test is acceptable.

Findings: Bifenox did not induce a detectable level of UDS in primary rat hepatocytes for an applied concentration range of 100 to 0.5 µg/ml. These treatments resulted in cell survival range of 63.7% to 99.8% at 20-23 hr after initiation of treatment. Treatment with 250 µg/ml was completely lethal.

The minimum criteria for UDS in this assay were a mean net nuclear grain count exceeding 6.85 or at least 12.7% of the nuclei containing 6 or more grains, or at least 2% of the nuclei containing 20 or more grains. All three criteria were clearly achieved for the 2 AAF positive control treatment. However, none of the treatments with test material caused nuclear labeling significantly different from the solvent control. The criteria for UDS were not even approached, and a dose-related response was not evident. The highest analyzed treatment (100 µg/ml) closely approached an excessively toxic treatment yet did not

result in nuclear labeling greater than the solvent control. Hence, the results of the assay were considered sufficient to evaluate bifenox as inactive in this test for genetic damage.

Conclusion: Bifenox was considered to be inactive in the primary rat hepatocytes UDS assay.

Mutagenic activity in Salmonella typhimurium with bifenox (Anonymous, 1983)

Findings and conclusion:

In this study, spontaneous reversion rate are unacceptable with strains TA 100 and TA 1535. Therefore, the study is rejected.

GLP status: no

Guideline: study is not fully conforming to directive 2000/32/EC Annex 4D, or directive 92/69/EEC method B14, directive 84/449 or OECD 471(1997-83) guideline.

Deviation from official protocol: strain TA102 or E. coli were not used (OK for directive 92/69); no positive control with S9 mix; single experiment. Phenotypic characteristics of strains were not checked. Only mean data are reported.

Material and methods: *Salmonella typhimurium* strains TA 100, TA1535 andTA1538 were exposed to bifenox (batch no 605, purity?) dissolved in DMSO using the plate incorporation assay. Five concentration (18, 54, 162, 486, 1398 µg/plate) were tested with and without rat liver Arochlor 1254 microsomes. Positive controls (MMNG and BaP) gave the expected results.

The study was not accepted in DAR (February 2006).

3.8.2 Animal data

3.8.2.1 CA 5.4.3 - *in vivo* genotoxicity studies

In order to assess the current reliability of the studies, the ToxRTool (Toxicological data reliability Assessment Tool)[1] has been used for evaluation. Consequently, these studies are categorized into Klimisch[2] category 1: reliable without restriction. Therefore, it is concluded that these *in vivo* studies are valid and are considered for classification.

Addressing versatile modes of action that may cause genotoxic effects, *in vivo* studies in mouse and rat bone marrow were conducted. The mouse micronucleus and metaphase analysis revealed no genotoxic potential, confirming the negative results from *in vitro* genotoxicity testing of the active substance Bifenox. A summary of the *in vivo* genotoxicity studies in somatic cells is presented in **Table 3.8.2-1.**

[1] The ToxRTool is the outcome of a project sponsored by the European Commission (CCR.IHCP.C433199.XO). The ToxRTool is property of the European Commission: © European Communities

[2] Klimisch et al., 1997, Regulatory Toxicology Pharmacology 25(1), 1-5

Table 3.8.2-1 Results of *in vivo* genotoxicity assays in somatic cells with Bifenox

Test	Test system	Test substance	Route of administration	Dose range tested	GLP / Guidelines	Result	Reference
Mouse micronucleus	Mouse bone marrow	Bifenox Batch # 20010903 Purity: 97.3%	Oral gavage	500, 1000, 2000 mg/kg bw	GLP / OECD 474	Negative	Anonymous, 2003 (DAR, 2006)
Metaphase analysis	Rat bone marrow	Bifenox Lot # 16230 Purity not stated	Oral gavage, 5 days	500, 1000, 1500 mg/kg bw	No GLP statement / assay was conducted according to standard procedures on which OECD 475 is based / test is judged to be	Negative	Anonymous, 1981 (DAR, 2006)

***In vivo* cytogenetics – mouse bone marrow cells bifenox** *(From April 2017 RAR)*

Previous evaluation

This study was evaluated in the original DAR and reviewed during the EU Review for **bifenox** and was considered to be acceptable. **New evaluation has been performed according AIR3 of Reg. (EC) No 1107/2009**. The study was performed with protocol corresponding to the method OECD guideline 474(1997-83). The study still meets the current data requirements.

Report	Anonymous 2003
Title	Micronucleus Test Of Bifenox Tech. In Bone Marrow Cells Of The Nmri Mouse By Oral Administration; LPT Lab. Of Pharmacology and Toxicology KG, Hamburg, Germany Feinchemie Schwebda GmbH
Document No	Report-no. 17124/1/03
Guideline	OECD guideline 474(1997-83)
GLP	Yes

Material and methods:

5 NMRI Crl: NMRI mice/sex/dose received by gavage, bifenox technical (20010903, 97.3%) suspended in 0.8% hydroxypropylcellulose at 500, 1000, 2000 mg/kg bw. Bone marrow was sampled at 24h (for all doses) and 48 h (for 2000 mg/kg bw). A preliminary test was performed at 30, 100, 300, 1000, 2000 mg/kg bw. At least 2000 cells were scored per animal where possible. Cyclophosphamide (27 mg/kg) was used as positive control and the sensitivity of the test was demonstrated.

Statistical analysis: inter-individual variations in n° MN PCE was estimated using a heterogeneity X^2 test.

Findings: No signs of systemic toxicity were noted after administration of bifenox up to the highest concentration of 2000 mg/kg bw. Hence for the main study, 3 doses were used (500, 1000 and 2000 mg/kg bw).

No bifenox related increase in micronucleated polychromatic erythrocytes was observed in the treated groups as compared to the corresponding negative reference item group at the 2 sampling times. The highest dose of 2000 mg/kg bw did not affect the ratio polychromatic to normochromatic erythrocytes. Cyclophosphamide treated mice had a significantly higher n° of MN PCEs.

Conclusions:

Under the present test conditions, bifenox technical, tested up to the highest reasonable dose of 2000 mg/kg bw showed no mutagenic properties in the mouse bone marrow micronucleus study at the 2 sampling times of 24 and 48 hr.

In vivo cytogenetics – mouse bone marrow cells bifenox – continuation *(From April 2017 RAR)*

Previous evaluation

This study was evaluated in the original DAR and reviewed during the EU Review for **bifenox** and was considered to be acceptable. **New evaluation has been performed according AIR3 of Reg. (EC) No 1107/2009**. The study was performed according OECD Test Guideline 475 corresponding to the test method B.39 of directive 2000/32/EC. Deviation from official protocol: Only 50 metaphases/rat were examined (in agreement with Dir 92/69, 84/449 or OECD guideline 1984). Animals were treated for 5 days. Mitotic index not measured. Samples were taken 6 hr after latest dose (in agreement with dir 84/449/EEC).

The study still meets the current data requirements. The study is accepted.

Report	Anonymous 1981
Title	Metaphase Analyses of rat bone marrow cells treated in vivo with bifenox technical; Mobil Enviromental and Health Science Lab., New Jersey, USA; Feinchemie Schwebda GmbH;
Document No	Report-no. 2312-80
Guideline	OECD guideline 475(1997-84)
GLP	No, only attest of QAU

Material and methods:

6 male Sprague-Dawley rats/dose received carbofuran in corn oil (98.3%) suspended in Methocel K4M at 1.5, 1, and 0.5 g/kg bw/ daily by gavage for 5 consecutive days. One animal was used for absorption study.

Colchicine was administered 4 hr after the last administration of test compound. Bone marrow cells from femur were collected 6 hr after the last administration. Criteria for evaluation of results are clearly reported. Cyclophosphamide (60 mg/kg bw, oral) was used as positive control. Fewer analyzable cells in positive control due to a technical error. Statistical analysis: X^2 analysis to compare test value to negative control.

Findings:

Bifenox technical did not induce any remarkable pharmacological effects. Cytotoxicity was not observed although bifenox was detected in blood. No clastogenic activity was seen with bifenox. Severe cytotoxicity was observed with cyclophosphamide, which was clastogenic.

Conclusions:

Bifenox technical did not significantly increase clastogenic events in rats bone marrow treated for 5 consecutive days at a daily oral dose of 500, 1000 or 1500 mg/kg bw.

3.8.3 Human data

No human data available on mutagenic properties from Bifenox.

3.8.4 Other data

3.8.4.1 Study 1 - CA 5.4.1 – data from open literature

Study reference

Report:	Sivikova, K., Dianovsky, J., 2000
Title:	Mitotic Index and Cell Proliferation Kinetics as Additional Variables for Assessment of Genotoxic Effect of The Herbicide Modown
Reference:	Acta Veterinaria Brno 69(1):45
Guidelines:	Not applicable
GLP	Not applicable

Detailed study summary and results:

Test substance and procedure

The *in vitro* effect of the herbicide Modown (with active component Bifenox) was tested for the ability to influence cell proliferation of PHA-stimulated bovine peripheral lymphocytes. Mitotic (MI) and proliferation (PI) indices were determined as an alternative for the screening of the cytostatic activity. The herbicide Modown exerted a clear effect on the inhibition of MI and PI over a concentration-tested range of 25 µg/ml to 1000 µg/ml. An expressive proliferation delays was found after the treatment with herbicide at a dose of 250 µg/ml ($P < 0.001$), while the higher doses of 500 and 1000 µg/ml had caused nearly complete mitotic inhibition in each donor ($P < 0.05$ and $P < 0.001$, respectively). A correlation between the PI and MI inhibition refers rather to cytostatic than cytotoxic effects of the herbicide. The results support the possibility of immunosuppression by herbicide exposure.

Modown consisting of 42% Bifenox from Rhone-Poulenc was dissolved in DMSO and was applied into culture flasks at 25, 50, 250, 500 and 1000 µg/mL for 24 and 48 h with lymphocytes from healthy donors for viability test. Whole blood cultures were cultivated for 72 h. Lymphocyte cultures were exposed to the herbicide for the las 24 h of cultivation. Colchicine was added 2 h before harvest. 100 metaphases per donor and concentration were analysed for determination of darkly stained chromatids, harlequin chromatids and past of metaphase with dark and light stained chromatids. Mitotic index was scored in total number of 2000 cells.

Results

A statistically significant inhibition of mitotic index (MI) in human lymphocytes was detected after treatment with the highest concentration of 5000 and 1000 µg/mL in both donors. The changes in cell cycle proportions were reflected in the proliferation delay. Modown induced a cell cycle delay over a concentration range of 25 to 500 µg/mL. The results demonstrate at all doses inhibitions of mitotic indices and proliferation indices suggesting cytotoxic effects.

3.9 Carcinogenicity

3.9.1 Animal data

3.9.1.1 Study 1 - CA 5.5 Long-term toxicity and carcinogenicity

Study reference

Report:	Anonymous (1987)
Title:	Bifenox potential tumorigenic and toxic effects in prolonged dietary administration to rats
Document No:	Rhone-Poulenc, 440137
Guidelines:	OECD 453 (1981), EC Directive 87/302 Annex V B.33
Deviations	Detailed daily observations reported for the first 4 weeks only. Ornithine decarboxylase and GGT were not measured.
GLP	Yes

Detailed study summary and results:

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool)[1] has been used for evaluation. Consequently, this study is categorized into Klimisch[2] category 1: reliable without restriction.

Therefore, it is concluded that this study is valid and is considered for classification.

Test substance, Test animals, Administration/exposure

In a 104-week combined carcinogenicity/long-term toxicology feeding study, 50 Charles River Sprague Dawley CD rats/sex/dose received Bifenox (Rhone Poulenc Agro, batch # 353-12-1, purity 98%) in their diet. The dose levels were set at 500, 1580, and 5000 ppm, corresponding to 18.9, 59, and 188 mg/kg bw/day for males, for females 24.6, 77, 252 mg/kg bw/day. A premix was prepared each week. Chemical analysis of the diet was carried out to confirm the homogeneity of the diet and stability of the test material. Four satellite groups of 20 males and 20 females were included for blood sampling at intervals and for interim sacrifice after 52 weeks of treatment.

Parameters evaluated during the study included mortality, clinical signs, body weight, food consumption, ophthalmoscopy, haematology, clinical chemistry, histopathology, organ weight neoplastic findings and mammary tumours.

[1] The ToxRTool is the outcome of a project sponsored by the European Commission (CCR.IHCP.C433199.XO). The ToxRTool is property of the European Commission: © European Communities

[2] Klimisch et al., 1997, Regulatory Toxicology Pharmacology 25(1), 1-55

Table 3.9.1-1a Further details for the incidence of islet cell tumors in background data from CD Sprague Dawley rats reported in Anonymous (1987) for the same test laboratory and strain from 1980 to 1982.

Study number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**			
Study start	Feb-80	Apr-80	Sep-80	Jun-80	Apr-81	Feb-81	Jul-81	Jan-82			
Animal supplier	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa		**% range**	
Study duration	115	115	105	108	104	104	105	104	**mean**	**Min**	**Max**
	Males										
Number of animals	100	100	50	50	100	50	100	50			
Number examined	100	96	50	49	100	50	97	50			
Islet cell tumours incidence	12	14	10	2	13	7	19	7			
%	12.0	14.6	20.0	4.1	13.0	14.0	19.6	14.0	**14.19**	**4.1**	**20.0**
	Females										
Number of animals	100	100	50	50	100	50	100	50			
Number examined	100	99	50	50	100	50	100	50			
Islet cell tumours incidence	5	4	5	4	3	2	7	3			
%	5.0	4.0	10.0	8.0	3.0	4.0	7.0	6.0	**5.51**	**3.0**	**10.0**

crusa: Charles River USA

See table below for data relating 1982 to 1984

Table3.9.1-2a Incidence of islet cell tumors in background data from CD Sprague Dawley rats for the same test laboratory and strain for the period 1982 to 1984

Study number	**8**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**			
Code number	cdr8201	cdr8203	cdr8207	cdr8207b	cdr8210a	cdr8210b	cdr8301	cdr8304	cdr8305	cdr8307	cdr8311	cdr8312	cdr8406	cdr8409	cdr8409	cdr8410a	cdr8410b			
Study start	Jan-82	Mar-82	Jul-82	Jul-82	Oct-82	Oct-82	Jan-83	Apr-83	May-83	Jul-83	Nov-83	Dec-83	Jun-84	Sep-84	Sep-84	Oct-84	Oct-84			
Animal supplier	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa	crusa		**% range**	
Study duration	104	111	104	104	110	106	105	105	110	106	106	106	106	106	106	106	106	**Mean**	**Min**	**Max**
	Males																			
Number of animals	50	105	60	50	50	50	50	55	50	50	50	55	100	50	50	50	55			
Number examined	50	104	60	50	50	50	49	55	50	49	50	55	100	50	50	50	55			
Islet cell tumours incidence	7	17	9	8	7	6	6	10	10	9	8	11	22	7	15	15	12			
%	14.0	16.3	15.0	16.0	14.0	12.0	12.2	18.2	20.0	18.4	16.0	20.0	22.0	14.0	30.0	30.0	21.8	**18.2**	**12**	**30**
	Females																			
Number of animals	50	105	61	50	50	50	50	55	50	50	50	55	100	50	50	50	55			
Number examined	50	105	61	50	50	49	50	55	50	50	50	55	100	50	50	50	55			
Islet cell tumours incidence	3	7	5	4	2	1	5	1	11	4	5	13	7	6	5	8	1			
%	6.0	6.7	8.2	8.0	4.0	2.0	10.0	1.8	22.0	8.0	10.0	23.6	7.0	12.0	10.0	16.0	1.8	**16**	**1.8**	**23.6**

crusa: Charles River USA

Results

No clear toxicological effects were observed in this study. Adverse findings are summarised in **Table 3.9.1-1.**

Tumours of the pancreas were not directly responsible for animal deaths. Islet cell adenoma and/or adenocarcinoma of the pancreas were observed for male rats at the low and intermediate dose reaching statistical significance when compared

with control. However, there was no evidence of a trend across the treatment groups. The low dose for females was found to have significantly more tumours than the controls, but again no trend across the treatment groups was apparent.

Table 3.9.1-1 Results of the Bifenox rat study, 104 weeks.

End point /dose	**0**		**500**		**1580**		**5000 ppm**	
Sex	**m**	**f**	**m**	**f**	**m**	**f**	**m**	**f**
Survival %	40	42	42	42	52	62	48	46
Bw gain week 0-26							↓6%	↓6%
Food consumption week 0- 26							(↓4%)	
Organ weight relative								
Liver interim sacrifice			(↑6%)	(↑9%)		(↑15%)	(↑6%)	(↑9%)
Macroscopic findings								
Masses pancreas	10/50	2/50	10/50	8/50	15/50	7/50	12/50	5/50
Skin masses	5/50		2/50		4/50		7/50	
Subcutaneous masses	8/50	37/50	9/50	29/50	11/50	36/50	16/50	33/50
Spleen enlargement		3/50		3/50		1/50		6/50
Uterus swelling		5/50		2/50		4/50		4/50
Histopathology: # affected rats / # examined								
Pancreas: Islet cell								
Adenocarcinoma								
Killed or dying during study	1/32	0/29	0/29	0/29	0/25	1/19	2/27	0/28
At terminal sacrifice	0/18	0/21	2/21	2/21	3/25	2/31	1/23	2/22
Total number adenocarcinoma	1/50	0/50	2/50	2/50	4/50	3/50	4/50	2/50
Adenoma								
Killed or dying during study	4/32	1/29	6/29	1/29	3/25	1/19	1/27	0/28
Terminal sacrifice	1/18	1/21	3/21	5/21	9/25	2/31	5/23	2/22
Total No adenoma	5/50	2/50	9/50	6/50	12/50	4/50	6/50	3/50
Total adenoma + carcinoma	6/50	2/50	11/50*	8/50*	15/50*	6/50	10/50	5/50
	12%	4%	22%	16%	30%	12%	20%	10%
S/C mass (benign)	6/50	26/50	5/50	24/50	7/50	27/50	13/50	20/50
Mammary fibroadenoma + focal epithelial atypia		0/50		2/50		7/50		1/50
Mammary fibroadenoma	1/50	22/50	0/50	23/50	0/50	27/50	1/50	15/50
Mammary adenoma		1/50						
Fibroma	2/50	2/50	0/50	2/50	3/50	5/50	4/50	5/50
Lipoma	5/50	4/50	4/50	0/50	4/50	0	6/50	3/50
S/C mass (malign)	1/50	7/50	3/50	6/50	1/50	15/50	2/50	9/50
Mammary adenocarcinoma		6/50		5/50		15/50		9/50

↑ ↓ statistically significantly different from control; ( ) not statistically significant
(T+D) terminal kill + during study kill
* statistically significant in pair-wise comparison

Discussion and conclusion

No statistical significance was found in the incidence of malignant islet cell tumours in any group of male or female rats when compared to control, nor was there any significant trend in malignant islet cell tumour incidence with increasing dosage. A statistical significance was found in a pair wise comparison for combined benign and malignant islet cell in male ($p = 0.05$) and female ($p = 0.03$) rats receiving 500 ppm and in male rats ($p = 0.04$) receiving 1580 ppm, but the dose response trend between control and exposure groups indicated a lack of statistical significance.

When comparing the combined incidence of tumours in the study with the historical control data based on adenoma and adenocarcinoma incidences provided by the laboratory (background data from the study report), the results at 500 and 1580 ppm are outside the concurrent historical control data. However, according to the open literature[1, 2, 3] the incidence of islet cell adenomas is within 1.67-25.71% and 1.43-14.29% for male rats and female rats (CD Sprague Dawley rats). For carcinoma the incidence is 0.77-14% and 0.77-4.29% for male and female rats respectively. Islet cell tumours subclassified as adenoma and adenocarcinomas, increase in incidence with age and are more frequently observed in males than in females.

Background data from the study report on islet cell tumours in CD Sprague Dawley rats was evaluated as demonstrated in **Table 3.9.1-2.**

Table 3.9.1-2 Incidence of islet cell tumors in background data from CD Sprague Dawley rats

Study number		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Incidence of islet cell tumors	Male	12 %	14 %	20 %	4 %	13 %	14 %	19 %	14 %
	Female	5 %	4 %	10 %	8 %	3 %	6 %	7 %	6 %

Table 3.9.1-3 Incidence of islet cell tumors in CD Sprague Dawley rats from open literature

Reference		**Gikins and Clifford, 2001**	**Majeed, 1997**	**Longnecker and Millar, 1990**
Species / Strain		Crl:CD Sprague Dawley	CD Sprague Dawley	CD Sprague Dawley
Incidence Islet tumours	Male	Adenocarcinoma: 0.8-14% Adenoma: 1.7-25.7%	11.7%	0-17%
	Females	Adenocarcinoma: 0.8-4.3% Adenoma: 1.4-14.3%	5.5%	

[1] Longnecker, D.S. and Millar, P.M., (1990) Pathology of tumours in laboratory animals. Vol I. Tumors of the rat, IARC scientific publications (99)

[2] Majeed, S.K., (1997) Studies of the incidence of spontaneous pancreatic tumors in ageing CD rats, Arzeneimittel-Forschung, 47 (7), 879-884

[3] Gikins, M.L.A., Clifford, C.B., (2001) Compilation of Spontaneous Neoplastic Lesions And Survival in Crl:CD (SD) BR Rats From Control Groups, Charles River Laboratories

Table 3.9.1-3a Elaboration of the public domain historical control data cited in connection with Anonymous et al., 1987.

Reference		**Lang, 1992** elaborated on instead of Gikins and Clifford, (2001)*	**Majeed, 1997**	**Longnecker and Millar, 1990**
Strain		Crl:CD Sprague Dawley, bred at Portland Michig Source Charles River Breeding Laboratories, Portage, Michigan, USA.	CD Sprague Dawley, (Charles River UK Ltd., Margate, Kent, UK)	CD Sprague Dawley ***Crl: COBS(r) CR(r) SD.*** These rats have been produced continuously at the Charles River Breeding Laboratories (CRBL) Wilmington, Massachusetts since 1955
Incidence Islet tumours	Male	Adenocarcinoma: 1.6-8.2% Adenoma: 2.9 -24.0%	11.7%	The reported incidence of islet cell tumours of rat has ranged from 0 to 17.6% in two small series of 71 male Sprague-Dawley Hap and 108 female
	Females	Adenocarcinoma: 1.4-8.2% Adenoma: 1.4-8.6%	5.5%	
Reference		Lang PL (1992) Spontaneous Neoplastic Lesions and Selected Non-neoplastic Lesions in the Crl:CD®BR Rat Published by Charles River Laboratories (February 1992)	Majeed, S. K. (1997). Studies of the incidence of spontaneous pancreatic tumours in ageing CD rats. Arzneimittel-forschung, 47(7), 879-884	Longnecker DS and Millar PM . (1994). Pathology of Tumours in Laboratory Animals. Vol. I. Tumours of the Rat. pp241-257 IARC Sci. Publ. No. 99, Publ. Lyon 1990 citing Anver MR, Cohen BJ, Lattuada CP, Foster SJ (1982) Age associated lesion in barrier-reared male Sprague-Dawley rate: A comparison between Hap:(SD) and Crl: COBS(r) CR(r) SD stocks. Exp. Agric. Res., 8(1), 3-22
Period covered		Studies with Start dates ranging from Dec 1985 to Feb 1989	1970 - 1995	Assumed to be 1980-1982.

***Although Gikins and Clifford, (2001) was cited, Lang, 1992 also published by Charles River Laboratories, is more temporally relevant.**

For this common type of tumour, the marginally significant increases at 500 and 1580 ppm in male rats results from a random occurrence of a low concurrent control rats.

For mammary adenocarcinoma, there was no significant trend in tumour incidence with increasing dose. The intermediate dose group however, showed a slight increase from the controls, but this difference did not attain statistical significance ($p = 0.08$). No significant treatment effects were found when the combined category of any mammary tumour was considered.

A NOAEL of 5000 ppm = 252 mg/kg bw/day in rats was set, because the effects were statistically not significant or lacked dose response.

3.9.1.2 Study 2 - CA 5.5 Long-term toxicity and carcinogenicity

Study reference

Report:	Anonymous (1982)
Title:	24-Month Carcinogenicity study in mice Bifenox (MCTR-1-79)
Document No:	Litton Bionetics Inc, 404836
Guidelines:	not fully conform to EEC 87/302 Annex V B.32 or OECD guideline 451 (1981)
Deviations:	Three females were pregnant; weekly observation instead of daily. Haematological test on 10 animals performed instead of blood smear of all animals.
GLP:	No

Detailed study summary and results:

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool)[1] has been used for evaluation. Consequently, this study is categorized into Klimisch[2] category 1: reliable without restriction. Therefore, it is concluded that this study is valid and is considered for classification.

Test substance, Test animals, Administration/exposure

In a 24-month carcinogenicity study, sixty B6C3F1 mice/sex/dose received Bifenox (Mobil Chem. Company, batch # 16230, purity 98.3%) in their diet. The dose levels were set at 50, 200 and 1000 ppm, corresponding to 7, 30 and 147 mg/kg bw/day for males and 9, 35, 179 mg/kg bw/day for females. The test diet was prepared monthly. Chemical analysis of the diet for homogeneity and correctness of formulation have agreed well with the limits set forth in the study protocol. At 12 and 24 months, 10 randomly selected mice/sex/dose were fasted overnight and sacrificed. A number of animals escaped or were withdrawn from the study. Parameters evaluated during the study included mortality, organ weight, clinical signs, body weight, food consumption, haematology, histopathology and neoplasms.

Results

In this study, the MTD was not reached. No clear toxic effects were demonstrated on mortality, clinical signs, body weight, food consumption or haematology through the course of the study. Adverse findings are summarised in **Table 3.9.1-4.**

Table 3.9.1-4 Effects observed in the mice study

End point /dose	0 ppm		50 ppm		200 ppm		1000 ppm	
	m	f	m	f	m	f	m	f
Survival %	70	60	73.3	58.3	68.3	56.6	70	63.3
Mean survival time (days)	649	626	642	614	631	636	611	632
Body weight week 104	n.a.	n.a.	↓7%	n.a.	↓9%	n.a.	↓7%	n.a.
Food consumption			No compound related effect					
Interim sacrifice: 12 month								
Haematology								
Leukocyte count:	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑33%	n.a.
RBC count	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑6%	n.a.

[1] The ToxRTool is the outcome of a project sponsored by the European Commission (CCR.IHCP.C433199.XO). The ToxRTool is property of the European Commission: © European Communities

[2] Klimisch et al., 1997, Regulatory Toxicology Pharmacology 25(1), 1-5

End point /dose	0 ppm		50 ppm		200 ppm		1000 ppm	
	m	f	m	f	m	f	m	f
Ht	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓4%
Relative organ weight:	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Liver	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑28%*	n.a.
Kidney	n.a.	n.a.	n.a.	n.a.	n.a.	↑13.5%	n.a.	↑21.3%
Terminal sacrifice								
Relative organ weight	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Kidney	n.a.	n.a.	n.a.	n.a.	n.a.	↑8%*	n.a.	↑18%*
Histopathology								
Non-tumours pathology - Scheduled + unscheduled death days 1-737								
N° examined mice	58	52	60	58	58	56	57	58
Kidney /cortex atrophy	0	2	2	0	1	2	6	4
Convoluted tubule hypertrophy	5	0	25	8	39	2	42	4
Urinary bladder multifocal inflammation	5	25	4	35	9	29	10	34
Liver inflammation	15	21	8	33	7	30	16	32
Adrenal cortex haemorrhage	0	0	0	0	0	1	0	5
Tumour pathology								
Scheduled + unscheduled death days 1-367								
N° examined mice	12	12	13	15	13	12	11	13
N° mice with tumours	1	0	0	2	0	0	2	2
Lung adenoma	1	0	0	0	0	0	1	0
Hepatocellular carcinoma	0	0	0	1	0	0	1	1
Haemopoietic system								
Malignant lymphoma	0	0	0	1	0	0	0	1
Scheduled +unscheduled death days 1-737								
N° examined mice	58	52	60	58	58	56	57	58
N° mice with tumours	25	17	20	22	24	27	29	27
Liver								
Hepatocellular adenoma	5	1	3	3	8	0	7	3
Hepatocellular carcinoma	4	1	9	0	6	0	11	2**
Haemangiosarcoma	4	0	1	0	0	0	0	1
Haemopoietic system								
Malignant lymphoma	4	6	2	12	4	12	5	13
Spleen haemangiosarcoma	0	0	1	0	0	1	2	3
Lungs								

End point /dose	0 ppm		50 ppm		200 ppm		1000 ppm	
	m	f	m	f	m	f	m	f
Adenoma	1	0	0	0	0	0	1	0
Metastatic	0	0	0	0	0	1	1	1
Alveolar/bronchiolar adenoma	2	3	2	0	4	2	2	1
Alveolar/bronchiolar carcinoma	2	1	4	0	3	3	3	1

* $p < 0.05$ as compared to controls : Dunnett's t test;
** weakly statistically significantly positive in female with Gehan-Breslow trend test
n.a. no data available

Hepatic neoplasms were diagnosed as carcinoma and adenomas and were encountered more frequently at 24 months in top dose group male mice (combined hepatocellular adenoma and carcinoma 31.57%), but this incidence was not unusually high for mice of this age and strain. Statistical analyses were performed which examined incidence of hepatocellular carcinoma, adenoma and carcinoma or adenoma in each sex separately. Of the tests employed, none indicated statistical significance at the $p < 0.05$ levels in males. In females the trend test was weakly positive when hepatocellular carcinomas alone were examined or when carcinomas were combined with adenomas ($p = 0.45$ and $p = 0.41$). Because of the small numbers of tumours involved, this finding is considered to represent a statistical aberration rather than evidence for oncogenicity. Anonymous et al (1990)[1] reported a mean incidence of 42.2% for male B6C3F1 control mice spontaneous liver tumours. Anonymous (1981)[2] report a mean incidence for five independent laboratories for spontaneous liver tumours in mal B6C3F1 mice of 32.1%.

Malignant lymphomas occurred more frequently in exposed than in unexposed females. In several instances, the line between hyperplasia and neoplasia was not clear. In no sex or dose group was the incidence of this class of tumour unusual for this age and strain of mouse.

Lung tumours were fairly frequent occurrences. The incidence of hepatic neoplasms in the males and malignant lymphomas in females as not unusual for this age and strain of mouse, statistical analysis provided no substantive evidence for oncogenicity

Haematology at 12 months was without significant observations. At terminal sacrifice reduced platelet counts were noted in males only which reached significance at the highest dose level. In females this parameter was unaffected or increased. Also at the highest dose level significantly reduced reticulocyte counts were noted in females while in males a non-significant reduction was noted. Please refer to **Tables 3.9.1-5** and **Table 3.9.1-6.**

Table 3.9.1-5 Haematological effects at terminal sacrifice - males

Dose	**0** ppm	**50** ppm	**200** ppm	**1000** ppm
Platelets [k/cmm]	10	9	10	10
	1578	1332	1298	**825***
	± 279	± 173	± 260	**± 46**
	-	↓16%	↓17%	**↓48%**
Reticulocytes (%)	10	11	10	10
	2.32	2.49	2.04	1.94
	± 0,24	± 0.11	± 0.11	± 0.06
	-	↑7%	↓12%	↓16%

* $p < 0.05$ as compared to controls: Dunnett's t test;

[1] Anonymus (1989), Spontaneous Neoplasm incidence in Fisher 344 Rats and B6C3F1 mice in two-year carcinogenicity studies: A national toxicology Program update, Toxicolic Pathology, 26 (3), 428-441

[2] Anonymus. (1981), Variability in the rates of some common naturally occurring tumors in Fischer 344 rats and (C57BL/6N X C3H/HEN) F1 (B6C3F1) mice, Journal of the National Cancer institute, 66 (6), 1175-1181

Table 3.9.1-6 Haematological effects at terminal sacrifice - females

Dose	0 ppm	50 ppm	200 ppm	1000 ppm
Platelets [k/cmm]	10	8	10	10
	734	1232*#	790	766
	± 97	± 211	± 17.4	± 57.8
	-	↑67%	↑7%	↑4%
Reticulocytes (%)	11	10	10	10
	2.51	2.54	2.26	**1.72***
	0.15	±0.19	±0.09	**±0.08**
	-	↑1%	↓10%	**↓31%**

\# not relevant - only 8 animals, one outsider at 2350
* $p < 0.05$ as compared to controls: Dunnett's t test;

Conclusion

Bifenox produced no direct evidence of oncogenicity. Taking into account the slight haematological effects seen at 1000 ppm at terminal sacrifice the overall NOAEL was 200 ppm (30 mg/kg bw/day).

3.9.1.3 Study 3 - CA 5.5 Long-term toxicity and carcinogenicity

Study reference

Report:	Anonymous (1992)
Title:	Review of the kidney slides from study: 24-month carcinogenicity study in mice with Bifenox
Document No:	Litton Bionetics Inc, 440135, Report n° R-21063,
Guidelines:	Not applicable
Deviations:	Not applicable
GLP:	Not applicable

In 1992, a large proportion of the slides of the kidney from male and female mice were reviewed. It is concluded, that diagnostic term i.e. “convoluted tubules, hypertrophy” used in the original report does not appear to be appropriate. A better term would have been “convoluted tubules, epithelial cell karyocytomegaly”. The grading system used is subjective and somewhat misleading. The review has shown, that the histological characteristics of the changes noted in the treated mice were the same as in controls, and there is no difference in the histological characteristics of the changes between the control animals and the treated ones. Treated female mice were affected with a very small incidence without any dose-related effect. Furthermore, no direct dose-effect relationship could be demonstrated. These changes were considered not to be of toxicological concern.

Reports from open literature

Reports that are available in open literature were evaluated to assess the long-term exposure effects from Bifenox, confirming low toxicologically adverse potential as assessed during long-term *in vivo* studies in rats and mice.

Data from open literature examined for spontaneous pancreas tumour occurrence in rats

Islet cell tumours (endocrine tumours), subclassified as adenoma and adenocarcinomas, increase in incidence with age and are more frequently observed in males than females. The incidence of spontaneous tumours has been reported by

different authors. The reported incidence of Islet cell tumours of rats has ranged from 0 to 17.6% in two small series of 71 male Sprague Dawley Hap and 108 females Sprague Dawley CD rats. The histological type of tumours of the Islet cell of the pancreas in rats is similar from that in humans, but in rats, the spontaneous incidence of Islet cell tumours is higher than that of exocrine tumours (Longnecker and Millar, 1990).

A survey of the incidence of spontaneous pancreatic tumours in CD rats from two-year carcinogenicity studies over a 15-year period was carried out. The survey revealed Islet cell adenomas to be the most common of pancreatic tumours with a higher incidence in untreated males (11.7% in comparison to females 5.5%) (Majeed, 1997).

A more recent compilation of spontaneous neoplastic lesions in Crl:CD (SD) BR rats from control groups (Charles River laboratories), in a total number of 1531 pancreases from male rats, 106 has Islet cell adenoma and 47 had carcinoma. In 1729 pancreases of female rats, 59 had Islet cell adenoma and 19 carcinoma. In this study minimum and maximum percent found were 1.67-25.71% and 1.43-14.29% for Islet cell adenoma in male and female rats, and 0.77-14% and 0.77-4.29% for carcinoma in male and female rats respectively (Giknis and Clifford, 2001).

In 2001, a study compared the effects of *ad libitum* (AL) overfeeding and moderate or marked dietary restriction (DR) on age related degenerative and proliferative changes of the endocrine pancreas in Sprague Dawley rats. In AL-fed rats, early changes in the islet morphology occurred, which resulted in a high incidence of islet fibrosis, focal hyperplasia and adenomas by two years. Compared to AL-fed rats, DR-fed rats had smaller pancreas, smaller pancreatic islets, smaller insulin secreting cell volumes, a lower degree of Islet fibrosis and a lower Islet cell BrdU labelling index, which correlated with a lower incidence of Islet adenoma and carcinoma at study termination. Moderate and marked degrees of DR delayed the onset and severity of Islet hyperplasia and fibrosis in a temporal and dose related manner (Anonymous 2001).

Overall conclusion on CA 5.5

In both rat and mice, no clear toxic effects were demonstrated on mortality, clinical signs, body weight, food consumption or haematology through the course of two long-term studies.

Bifenox is not carcinogenic in rats or mice. This conclusion is supported by the absence of genotoxic activity of Bifenox and published data on background tumour incidences.

Additional information on CA 5.5: Details on Reliability Evaluation of studies cited but not included

In order to assess the current reliability of the studies that were already submitted in the context of the inclusion of the active substance Bifenox in Annex I of the Council Directive 91/414/EEC, the ToxRTool (Toxicological data reliability Assessment Tool)[1] has been used for evaluation. The ToxRTool is designed to assess the inherent quality of toxicological data as reported in a publication or test report.

The following table shows the evaluation scores and overall sums for criteria assigned in the studies that were conducted on Bifenox long-term toxicity. Categorization resulting from the sum of the scores is provided at the end of the table.

Table 3.9.1-8 Scoring and evaluation of studies for Bifenox long-term toxicity and carcinogenicity

	Anonymous 1987	Anonymous 1982

[1] The ToxRTool is the outcome of a project sponsored by the European Commission (CCR.IHCP.C433199.XO). The ToxRTool is property of the European Commission: © European Communities

GLP status of the studies		Yes	Yes
Criteria Group I: Test substance identification		**Score**	
1	Was the test substance identified? *	1	1
2	Is the purity of the substance given?	1	1
3	Is information on the source/origin of the substance given?	1	1
4	Is all information on the nature and/or physico-chemical properties of the test item given, which you deem indispensable for judging the data?	1	1
Criteria Group II: Test organism characterization			
5	Is the species given?	1	1
6	Is the sex of the test organism given?	1	1
7	Is information given on the strain of test animals plus, if considered necessary to judge the study, other specifications?	1	1
8	Is age or body weight of the test organisms at the start of the study given?	1	1
9	For repeated dose toxicity studies only: Is information given on the housing or feeding conditions?	1	1
Criteria Group III: Study design description			
10	Is the administration route given? *	1	1
11	Are doses administered or concentrations in application media given? *	1	1
12	Are frequency and duration of exposure as well as time-points of observations explained? *	1	1
13	Were negative and positive controls included? *	1	1
14	Is the number of animals per group given? *	1	1
15	Are sufficient details of the administration scheme given to judge the study?	1	1
16	For inhalation studies and repeated dose toxicity studies only: Were achieved concentrations analytically verified or was stability of the test substance otherwise ensured or made plausible?	1	0
Criteria Group IV: Study results documentation			
17	Are the study endpoint(s) and their method(s) of determination clearly described?	1	1
18	Is the description of the study results for all endpoints investigated transparent and complete?	1	1
19	Are the statistical methods applied for data analysis given and applied in a transparent manner?	1	1

Criteria Group V: Plausibility of study design and results			
20	Is the study design chosen appropriate for obtaining the substance-specific data aimed at? *	1	1
21	Are the quantitative study results reliable?	1	1
Overall Sum		21	20
Numerical result leads to initial Category:		**1**	**1**
Checking grey scores* leads to revised Category:		**1**	**1**

* Some criteria (marked red in the tool, here highlighted grey) have special importance and are necessary for Cat. 1 or Cat. 2. Those points are evaluated with special care (i.e. test substance identification or species)

** Explanation: One point is also given if criterion is not necessary, applicable or required

3.9.2 Human data

No human data available on carcinogenic properties from Bifenox.

3.9.3 *In vitro* data (e.g. in vitro germ cell and somatic cell mutagenicity studies, cell transformation assays, gap junction intercellular communication tests)

In vitro studies on the genotoxic and mutagenic potential did not reveal any indication of genotoxicity. No further *in vitro* data are available on carcinogenic properties from Bifenox.

3.9.4 Other data (e.g. studies on mechanism of action)

No other data are required on Bifenox.

3.10 Reproductive toxicity

3.10.1 Animal data

3.10.1.1 Study 1 - CA 5.6.1 Generational studies

Study reference

Report:	Anonymous (1995)
Title:	Bifenox two generation reproduction study in rats
Document No:	Inveresk Research International, 600885
Guidelines:	Not fully compliant to OECD 416
Deviations	Mating procedure limited to 1 week (+ 2 weeks with another male if necessary)
GLP	Yes

Detailed study summary and results:

Test substance, Test animals, Administration/exposure

In a two-generational study 28 Sprague Dawley rats of Charles River CD strain/sex/dose received Bifenox at 125, 750 and 4500 ppm in their diet Bifenox (Rhone Poulenc, Pont-le-Claix, batch # 9401021, purity 99.2%). 24 male and female F_1 weanlings were selected for rearing to maturity and mating to produce F_2 generation. Analysis of formulated diet showed, that the concentrations were within ± 10% of nominal concentration at all dose levels and diets were homogenous.

Results

Requested maternal body weight data, fertility and developmental parameters are provided as far as they were available in the report, in the three tables below. With respect to providing corrected maternal body weights, there were no gravid uterine weights provided in the 2 generation study report, however body weight gains for various periods have been presented as a % proportion of the starting bodyweights for the period in question (highlighted rows).

No historical control data was cited against findings from this study in the evaluation that would require further elaboration.

Table 3.10.1-1a: Parameters of maternal toxicity in the two-generation rat study with bifenox. Anonymous (1995): maternal toxicity. Blank cell = zero incidence

Generation	Dose Level	0 ppm	125 ppm	750 ppm	4500 ppm
		Mortality			
F0		1			
F1				1	
		Clinical observations and Necropsy findings			
F0	Hair Loss / scabbing	3	6		2
	Red liquid evident from Vagina				1
	Ear torn/black/encrusted				1
	Mass (no further info provided)				1
	Uterus:dilated	2	2		
	Vagina:mass	1			
	Cervix: enlarged		1		1
	Lungs: dark				1
F1	Scabbing/staining/coat scruffy	1		2	1
	Lump/mass	1		1	
	Hair loss	4	1		2

	Pale/breathing difficulty			1	
	Eyelid encrusted/lacrimation		1	3	
	Red liquid from vagina/nervous/ agitated/piloerection			1	
	Incisors short/chipped				1
	Hind limbs swollen/purple/red	1			
	Uterus dilated	1		1	
	Red staining around mouth and nose/lungs dark/blood in thoracic cavity			1	
		Maternal body weight, body weight gain and food consumption			
F0 premating	Body weight (g±SD) Week 0	142±14	140±12	145±13	140±14
	Weight gain (g) Week 0-9	126	132 (+5%)	132 (+5%)	117 (-7%)
	Weight gain, Week 0-9, corrected for maternal body weight by expression as a percentage of starting body weight (%change versus control)	88.7%	94.3% (+6.3%)	91.0% (+2.6%)	81.4% (-8.2%)
	Total Food consumption Week 0-9 (total g/animal)	1119	1348	1343	1295
	Food conversion to body weight (% w/w)	11.3	9.8	9.8	9.0
F1 premating	Body weight (g±SD) Week 3	69±15	67±16	75±16	56±10
	Weight gain (g) Week 3-15 (%change vs control)	227	238 (+5%)	236 (+4%)	220 (-3%)
	Weight gain, Week 3-15, corrected for maternal body weight by expression as a percentage of starting body weight (%change versus control)	329.0%	355.2% (+8.0%)	314.7% (-4.4%)	392.9% (+19.4%)
	Total Food consumption Week 3-15 (total g/animal)	1782	1811	1816	1716
	Food conversion to body weight (% w/w)	12.7	13.1	13.0	12.8
F0 gestation	Body weight (g±SD) D0	305±30	318±34	319±30	298±30
	Weight gain (g) D 0-20	156	154 (-1%)	147 (-6%)	137 (-12%)
	Weight gain, D 0-20, corrected for maternal body weight by expression as a percentage of starting body weight (%change versus control)	51.1%	48.4% (-5.3%)	46.1% (-9.9%)	46.0% (-10%)
	Total Food consumption D 0-20 (total g/animal)	613	653	630	620
	Food conversion to body weight (% w/w)	2.0	2.1	2.0	2.1
F1 gestation	Body weight (g±SD) D0	297±28	299±25	301±30	275±30
	Weight gain (g) D 0-20	157	157	147 (-5%)	147 (-6%)

	Weight gain, D 0-20, corrected for maternal body weight by expression as a percentage of starting body weight (%change versus control)	52.9%	52.5% (-0.7%)	48.8% (-7.6%)	53.5% (+1.1%)
	Total Food consumption D 0-20 (total g/animal)	629	650	608	638
	Food conversion to body weight (% w/w)	25.0	24.2	24.2	23.0
F0 lactation	Body weight (g±SD) D 0	337±38	344±39	346±32	329±39
	Body weight gain (g) D 0-21	10	16	11	18 (+5%)
	Weight gain, D 0-21, corrected for maternal body weight by expression as a percentage of starting body weight (% change versus control)	3.0%	4.7% (56.7%)	3.2% (7%)	5.5% (84.4%)
	Total Food consumption D 0-21 (total g/animal)	1534	1544	1583	1455
	Food conversion to body weight (% w/w)	0.65	1.04	0.69	1.24
F1 lactation	Body weight (g±SD) D 0	332±37	339±34	336±40	325±40
	Body weight gain (g) D 0-21	9	7	20	14
	Weight gain, D 0-21, corrected for maternal body weight by expression as a percentage of starting body weight (%change versus control)	2.7%	2.1% (-23.8%)	6.0% (+119.6%	4.3% (+58.9%)
	Total Food consumption D 0-21 (total g/animal)	1461	1517	1525	1454
	Food conversion to body weight (% w/w)	0.6	0.5	1.3	1.0

Table 3.10.1-1b: Fertility, gestational and birth indices and pup bodyweight parameters

Dose Level	**0 ppm**	**125 ppm**	**750 ppm**	**4500 ppm**
	F0 Dams Fertility, gestational and birth indices and pup bodyweight parameters			
Median number of nights to positive mating sign	2	2.5	2.5	2
Number passing one oestrus	0	0	1	1
Male fertility Index (%)[a]	68	75	79	86
Female fertility index (%)[a]	71	82	82	86
Duration of gestation				
21 days	2	5	3	5
22 days	17	16	16	19
23 days	1	2	4	0
Mean duration gestation	22.0	21.9	22.0	21.8
Gestation index[b] % dams producing live litters (N)	100 (20)	100 (23)	100 (23)	100 (24)
Mean implant sites±SD	17.2±3.3	17.3±1.4	17.5±1.3	16.1±2.6
Mean pups born/litter ±SD	15.5±3.4	15.8±1.7	15.4±2.6	14.8±2.5

	Mean live pups ±SD			
Day 0 lactation	15.4±3.4	15.6±1.8	15.1±2.8	14.6±2.4
Day 4 lactation	13.8±3.3	14.4±2.9	14.7±2.7	14.4±2.5
Day 21 lactation	13.6±3.3	13.3±3.1	13.8±2.8	13.0±3.4
	F1 pup indices			
	Birth index[c]			
Mean litter index (%)	90	90	88	92
Number losing >2 pups	3	6	6	4
Number of litters	20	23	23	24
	Live Birth index[d]			
Mean litter index (%)	99	99	97	99
Number losing >1 pups	0	1	2	1
Number of litters	20	23	23	24
	Viability index Day 0-4[e]			
Mean litter index (%)	91	86	89	92
Number losing >3 pups	4	4	4	2
Number of litters	20	23	23	24
	Lactation index Days 4-21[f]			
Mean litter index (%)	98	94	99	98
Number losing >1 pups	1	2	1	2
Number of litters	20	23	22	24
	Overall survival index Birth to Day 21[g]			
Mean litter index (%)	89	81	86	88
Number losing >4 pups	3	4	4	2
Number of litters	20	23	23	24
	Group mean litter weight (g±SD)			
Lactation Day 1	97±20	93±17	97±17	92±15
Lactation Day 21	621±118	604±104	635±108	478±96***
	Mean litter pup weight (g±SD)			
Lactation Day 1 males	6.7±0.9	6.6±0.7	6.8±0.7	6.5±1.0
Lactation Day 21 males	48.6±9.2	48.2±9.7	47.6±6.5	38.9±6.4***
Lactation Day 1 females	6.3±0.9	6.3±0.7	6.5±0.6	6.2±0.7
Lactation Day 21 females	46.0±9.0	46.2±9.2	46.3±5.4	36.9±5.9***
	F1 Dams Fertility, gestational and birth indices and pup bodyweight parameters			
Median number of nights to positive mating sign	3	3	3	2.5
Number passing one oestrus	0	0	0	3
Male fertility Index (%)[a]	92	88	83	88
Female fertility index (%)[a]	92	92	92	92
Duration of gestation				
21 days	5	5	4	11
22 days	17	16	16	10
23 days	0	0	2	1
Mean duration gestation	21.8	21.8	21.9	21.5
Gestation index[b] % dams producing live litters (N)	100 (22)	100 (22)	100 (22)	100 (22)
Mean implant sites±SD	15.8±2.8	15.8±2.6	16.3±2.7	15.1±2.1

Mean pups born/litter ±SD	14.3±2.9	14.7±2.6	14.3±2.7	13.2±2.0
	Mean live pups ±SD			
Day 0 lactation	14.3±2.9	14.5±2.6	14.1±2.5	13.2±2.1
Day 4 lactation	13.6±2.9	13.2±3.3	13.6±2.5	12.9±2.3
Day 21 lactation	13.3±2.8	12.9±3.2	13.4±2.6	12.4±2.2
	F2 pup indices			
	Birth index[c]			
Mean litter index (%)	91	93	89	89
Number losing >2 pups	6	3	6	4
Number of litters	22	22	21	22
	Live Birth index[d]			
Mean litter index (%)	100	99	99	99
Number losing >1 pups	0	0	1	0
Number of litters	22	22	21	22
	Viability index Days 0-4[e]			
Mean litter index (%)	91	92	89	84
Number losing >3 pups	0	3	2	3
Number of litters	22	22	22	22
	Lactation index Days 4-21[f]			
Mean litter index (%)	98	98	98	96
Number losing >1 pups	0	1	1	2
Number of litters	21	21	19	19
	Overall survival index Birth to Day 21[g]			
Mean litter index (%)	89	89	81	80
Number losing >4 pups	0	3	1	3
Number of litters	22	21	20	22
	Group mean litter weight (g±SD)			
Lactation Day 1	90±16	94±15	94±15	86±16
Lactation Day 21	604±100	599±113	628±78	470±65***
	Mean litter pup weight (g±SD)			
Lactation Day 1 males	6.7±0.7	6.8±0.8	7.0±0.8	6.7±0.5
Lactation Day 21 males	47.7±7.6	48.9±8.0	48.9±7.9	39.3±5.1***
Lactation Day 1 females	6.3±0.7	6.7±1.7	6.6±0.7	6.4±0.5
Lactation Day 21 females	45.3±7.2	46.5±7.7	46.9±7.5	37.5±4.9***

*** Statistically significantly different from controls (P<0.001)
a = Number of pregnant females or siring males / number paired
b = Number bearing live pups / number pregnant
c = Total number of pups born (live and dead) / Number of implantation scars
d = Number of pups live on Day 0 of lactation / Total number born
e = Number of pups live on Day 4 of lactation / Number live on Day 0
f = Number of pups live on day 21 of lactation / Number live on Day 4
g = Number of pups live on Day 21 of lactation / Total number of pups born (live and dead)

Table 3.10.1-1c: Developmental toxicity and other findings in pups

Dose Level	**0 ppm**	**125 ppm**	**750 ppm**	**4500 ppm**
	F1 pups			
Litters (pups) with malformations	**0**	**1 (2)[a]**	**0**	**1(1)[d]**

Litters (pups) with other findings (but no malformations)	**1(1)[c]**	**0**	**1 (1)[b]**	**1(2)[e]**
	F2 pups			
Litters (pups) with malformations	**0**	**0**	**0**	**0**
Litters (pups) with other findings (but no malformations)	**0**	**0**	**0**	**2(2)[f,g]**

[a]Two pups in F0parent-F1Litter 152 (125 p.p.m. Bifenox), died shortly after birth, with multiple abnormalities including misshapen cranium, shortened lower jaw, open eyes, cleft palate, fused digits and subcutaneous oedema.
[b]One pup in F0parent-F1Litter 180 (750 p.p.m. Bifenox) with a small kidney at necropsy.
[c]One pup in F0parent-F1Litter 139 (Control), killed Day 19, with body tremors, piloerection, encrusted eyes and apparent hind limb weakness/ataxia.
[d]One pup in F0parent-F1Litter 212 (4500 p.p.m. Bifenox), killed Day 23, with ataxia, hydrocephalus and one eye apparently absent.
[e]One pup in F0parent-F1Litter 223 (4500 p.p.m. Bifenox), died Day 19, with piloerection prior to death. Second pup with piloerection and swollen abdomen Day 21.
[f]One pup in F1parent-F2Litter 479 (4500 p.p.m. Bifenox), killed Day 16, with a firm, lobular mass on the lower lip.
[g]One pup in F1parent-F2Litter 496 (4500 p.p.m. Bifenox), small with brown fluid in one kidney at necropsy.

In this study, the MTD was not reached. Two rats died during the course of the study, without association to treatment. Clinical signs and necropsy findings did not indicate any association with treatment.

At the highest dose, the body weight of F_0 males and females was slightly reduced prior to pairing. In the F_1 generation, body weight after weaning was lower than control. During gestation, mean weight gain of females at 4500 ppm was lower in both generations. Food consumption was slightly affected in both sexes, but considered too small to be attributable to treatment.

Mating performance, fertility and duration of gestation were not considered to be affected by treatment. Litter size, pup survival and mean number of implantation was slightly reduced at 4500 ppm in both generations. The differences did not reach statistical significance and were considered incidental.

During the lactation period, the mean body weight gain of litter and pups was lower at top dose and on day 21 weights were approximately 80% of control. The reduction in litter and pup weights at 4500 ppm were statistically significant lower than controls but were accompanied by (slight) parental toxicity (evidenced by reduced body weight gain at top dose). Based on this, these effects were not considered to be relevant for a classification with regard to reproduction toxicity. Fertility parameters were not affected. There are no hints for a dose-relationship for effects on the offspring. In contrast, in the parental generation females reveal slight reduction of body weight gain even in the mid dose during gestation (up to 12%) and especially a significant reduction of body weight gain during lactation from Day 1 to Day 14 (up to 42%). The NOAEL is based on statistically significant reduction of litter/pup weight at top dose, clearly evident at slight parental toxicity and was also not considered to be relevant for a classification in view of the accompanying parental toxicity. Abnormalities among pups did not suggest any association with treatment. Organ weight was not affected by treatment, histopathology was not performed.

Conclusion

A reproductive NOAEL = 750 ppm (148 mg/kg bw/day) was based on decreased pup and litter weight in F_1 and F_2 generation at 4500 ppm. The reproductive effects occurred in the presence of slight parental (systemic) toxicity as suggested by the decreased body weight gain seen at 4500 ppm. NOAEL parental toxicity = 750 ppm (44.5 mg/kg bw/day).

3.10.1.2 Study 2 – CA 5.6.2 Developmental toxicity

Study reference

Report:	Anonymous (1987)
Title:	Effect of Bifenox on pregnancy of the rat
Document No:	Huntington Research Centre, 412538
Guidelines:	Not fully compliant to OECD 414
Deviations:	Low number of gravid females in control group (17/25) and top dose group (15/25).
GLP:	Yes

Detailed study summary and results:

Test substance, Test animals, Administration/exposure

25 female Crl: COBS CD (SD) BR strain rats/dose received by gavage Bifenox (Rhone Poulenc Agrochemicals, batch # 353-12-1, purity 98%) as a suspension in 1% aqueous methylcellulose. The dose levels were set at 225, 900 and 3600 mg/kg bw/day on days 6 - 15 of gestation. Dosages for the main study were based on a preliminary study in which treatment at 3000 mg/kg bw/day was associated with an apparent suppression of maternal weight gain. Formulations were prepared daily.

Results

Data available in the report pertaining to maternal toxicity is provided in the table below.

The extra details on the historical control data presented were requested from current incarnation of the test laboratory. The laboratory could not provide exact study details to the data cited, but did state that: "The HCD presented in the report would have been matched to the study for species, strain, supplier and performing laboratory."

Table 3.10.1-1d: Maternal toxicity findings and key fetal findings including those for which historical control data was cited in the report, for the developmental rat toxicity study by Anonymous (1987)

Endpoint/dose (mg/kg bw/d)	**0**	**225**	**900**	**3600**
N° mated females	25	25	25	25
Mortality	0	0	0	5
Not pregnant	7	5	3	5
Clinical signs	none	none	none	Salivation, staining mouth, patchy hair loss
	Water consumption (g/animal/day)			
D 13	27	35	33	38
D 14-16	32	37	37	42
D 17-19	33	39	40	38
Avg D 13-19 (% change vs control)	27.9	37.6 (+35%)	37.7 (+35%	39.7 (+42%)

	Food consumption (g/animal/day)			
D 6-9	21	20	20	19
D 10-13	24	25	25	23
D 14-16	25	26	26	25
D 17-19	25	26	26	27
Avg D 6-19	23.6	24.1	24.2	23.4
Body weight (g)				
D 6	228.9	231.5	230.2	235.7
D 20	345.8	348.0	343.0	351.3
Body weight gain (g) D 6-20	116.9	116.5	112.8	115.6
Maternal Reproductive performance				
Total resorptions			1	
N° females with live young	17	20	21	15
N° corpora lutea	13.1	13.4	13.6	13.5
N° implants	12.3	12.3	12.2	12.8
N° dead implants	0.9	0.5	1.1	1.1
Pre implantation loss %	6.1	10	9.1	4.8
Post implantation loss %	7.8	3.7	11.4	8.5
Mean litter weight (g)	39.19	40.43	38.29	40.64
Mean fetal weight (g)	3.45	3.41	3.5	3.46
Fœtal examination:				
N fetuses (litters) examined	98 (17)	119 (20)	115 (21)	88 (15)
	Skeletal evaluation: % fetal incidence (N litters affected)			
Number of ribs				
12/12	-	-	-	1.1 (1)
13/13	98.0 (17)	98.8 (20)	95.6 (21)	92.2 (15)
13/14	-	0.6 (1)	0.8 (1)	5.3 (3)
14/14	1.0 (1)	1.6 (2)	-	1.1 (1)
Historical control data extra 14th rib:	10 studies performed in 1985: mean fetal incidence: 2.9% with a range of 0.9-7.1%			
Small fontanelles	2.0 (1)	2.1 (2)	3.6 (3)	1.1 (1)
Medium fontanelles	96 (17)	95.3 (20)	94.4 (21)	88.8 (15)
Large fontanelles	2 (2)	2.6 (3)	2 (3)	10.1 (6)

Frontonasal suture enlarged	1.0 (1)	5.4 (4)	2.6 (3)	1.3 (1)
Incomplete ossification of frontal/parietal/squamosal/jugal/nasal bone (s)	1.0 (1)	3.3 (4)	1.0 (1)	3.2 (3)
Incomplete ossification of interparietal bone	5.1 (5)	16.7 (12)	12.4 (7)	12.1 (5)
Incomplete ossification of supraoccipital bone	8.0 (6)	11.5 (11)	6.2 (7)	7.2 (5)
Patchy/incomplete ossification of one or more cranial bones	1.0 (1)	0.7 (1)	-	-
Incomplete ossification/absence of hyoid bone	7.0 (6)	14.0 (12)	12.2 (11)	14.4 (8)

The results reported in the table are not statistically significant.

Maternal data: At top dose, one rat died and four were sacrificed *in extremis*. Autopsy indicated that at least three of these cases may have resulted from difficulty in dosing caused by the highly viscous nature of the suspension. Clinical signs were noted at top dose (salivation, staining of mouth, patchy hair loss) which show that this dose was clearly maternal toxic. Water consumption was increased among all treated groups compared to control group. At 3600 mg/kg bw/day, food consumption appeared to be reduced during the initial four days of treatment and marginally increased during the post-treatment period although body weight gain during pregnancy was essentially similar for all groups. Necropsy findings did not reveal any compound related effects. Treatment with Bifenox had no apparent effect on live litter size, implantation loss or litter and foetal weight.

Litter data: At 3600 and 900 mg/kg bw/day there was a marginally higher incidence of foetuses with an extra 14th rib, at 3600 mg/kg bw/day a marginally higher incidence of foetuses with a large fontanelle compared to controls. Incomplete ossification of the intraparietal and/or hyoid bones which had a higher incidence among treated groups, showed no dose-related trends and these minor differences from concurrent controls were considered too minimal to be attributable to treatment with Bifenox.

Discussion and conclusion

The findings extra 14th ribs, fontanelle enlargement and incomplete ossification/absence of hyoid bone will be discussed in more detail. The incidences of these findings are summarized in the following table.

Table 3.10.1-1 Selected findings in the rat developmental toxicity study

	Finding in % fetal incidence (number of litters affected)			
Dose (mg/kg bw/day)	**0**	**225**	**900**	**3600**
Number of fetuses (litters) examined	95 (17)	118 (20)	118 (21)	88 (15)
Size of fontanelle				
small	2.0 (1)	2.1 (2)	3.6 (3)	1.1 (1)
medium	96.1 (17)	95.3 (20)	94.4 (21)	88.8 (15)
large	2.0 (2)	2.6 (3)	2.0 (3)	10.1 (6)
Incomplete ossification/absence of hyoid bone	7.0 (6)	14.0 (12)	12.2 (11)	14.4 (8)
Number of ribs				
12/12	-	-	-	1.1 (1)
13/13	98.0 (17)	98.8 (20)	95.6 (21)	92.2 (15)
13/14	2.0 (2)	0.6 (1)	3.6 (3)	1.3 (1)
14/14	-	0.6 (1)	0.8 (1)	5.3 (3)
Historical incidence 14th rib (min – max)	0.9 - 7.1 %			

The extra 14th rib incidences were within the historical data range given in the report (up to 7.1 %) so that they are due to variability and not to treatment. Furthermore, the other findings of extra 13th and combined 13th and 14th ribs were not dose-related and thus most likely due to variation which supports an absence of an effect of Bifenox on the rib development.

The incidences of small, medium and large fontanelles show a slight increase of large fontanelles in the highest dose. However, the size definitions are not explained and can only be subjective so that this increase is of doubtful relevance and appears to be likely only a result of a subjective size shift between medium and large. Since this increase occurred at the highest dose only which showed strong maternal toxicity a size reduction might rather be expected at this maternal-toxic dose and not an enlargement. However, the incidence of small size fontanelles was not increased by treatment. Thus, this finding is not supported by the overall incidences of the different fontanelle sizes and therefore not regarded as relevant for human safety.

The incidences of the finding incomplete ossification/absence of hyoid bone did not show a dose-response relationship, since the incidence in the low dose was as high as that of the highest dose, whereas the incidence in the 900 mg/kg bw group was lower again despite a 4-fold higher dose compared to the low dose.

Overall, therefore, the discussed findings in the rat developmental toxicity study are not evidence of a treatment effect but are rather the consequence of variability within the normal background.

Not taking into account the increased water consumption reported at all dose levels, which was considered not to be treatment related, a NOAEL maternal was proposed at 900 mg/kg bw/day, based on mortality and clinical signs occurring at 3600 mg/kg bw/day.

The developmental NOAEL was 900 mg/kg bw/day, taking into account the marginally higher incidence of foetuses/litters with a large fontanel at 3600 mg/kg bw/day compared to controls.

3.10.1.3 Study 3 - CA 5.6.2 Developmental toxicity

Study reference

Report:	Anonymous (1986)
Title:	Developmental toxicity (embryo/fetal toxicity and teratogenic potential) study of Bifenox technical administered orally (stomach tube) to New Zealand white rabbits
Document No:	Argus Research Laboratories, Inc. 413657
Guidelines:	Not fully compliant to OECD 414
Deviations	Dose selection: Two doses without effect, highest dose inducing mortality
GLP	Yes

Detailed study summary and results

Test substance, Test animals, Administration/exposure

In a teratology study in rabbits, 20 female New Zealand white [Hra;(NZW)SPF] rabbits per dose received Bifenox (batch n° 312165, purity 98.2%) in aqueous 0.5% carboxymethyl cellulose at 2, 20 and 200 mg/kg bw/day by gavage on days 6-18 of gestation. The rabbits were evaluated for clinical signs, mortality, body weight gain and feed consumption. On gestation day 29 all surviving pregnant does were sacrificed and necropsied. Foetuses were delivered by Caesarean section, weighed and examined for gender and external, visceral and skeletal alterations.

Results

In the rabbit studies at the top dose there were maternal deaths, gastric ulcerations, perturbations to the condition of the faeces, and resorptions and abortions indicating that this dose produced severe toxicity.

Data available in the report pertaining to maternal toxicity is provided in the two tables below (Table 3.10.1-1a, Table 3.10.1-1b).

The extra details on the historical control data are presented in the table Table 3.10.1-1c.

Table 3.10.1-1a: Maternal toxicity findings and key fetal findings including those for which historical control data was cited in the report for the developmental rabbit toxicity study by Anonymous (1986).

Endpoints/ dose	**0**	**2**	**20**	**200 mg/kg bw/d**
	Mortality and clinical signs			
Mortality				3[a]
Dried feces	2/13	0/0	2/11	8**/41**
No feces present	1/2	0/0	1/1	2/9**
Soft or liquid feces	1/1	3/8c	8**/18**	1/3
Alopecia	3/18	4/19	8/52**	3/21
	Necropsy observations			
Aborted	0	0	0	3
Gastric ulceration[b]	0	0	0	6
Kidneys light brown in colour	0	0	0	1
Kidney cortex light brown in colour	0	0	0	1
Liver pale brown in colour	0	0	0	1
Urine red brown in colour	0	0	0	1
Left uterine horn filled with dark red fluid	0	0	0	1
	Bodyweight and bodyweight gain			
Body weight (kg) Day 29	4.27±0.46	4.25±0.41	4.38±0.50	4.12±0.52
Body weight gain (kg) Day 6-29	0.30±0.18	0.23±0.19	0.30±0.14	0.13±0.38 (↓ Day 6-12)
Food consumption Day 6-29 (g/animal/day)	146.1±30	147.1±33.3	147.9±34.8	135.2±39.7
Food conversion to body weight (% w/w)	0.21	0.16	0.20	0.10
	Reproductive data			
N° gravid females	20	20	20	20
Pregnant rabbits (n°)	17	16	20	16
Corpora lutea mean	9.8	11	10.7	9.4
Implantations mean	7.2	7	7.9	7.8
Litter size mean	6.6	6.6	7.5	6.8
N° live/death fetuses	106/0	106/0	143/0	75/0
N°early/late resorption	8/2	5/1	5/2	8/3
Resorptions mean	0.6±1.3	0.4±0.5	0.4±0.8	1±1.4
Live fetal bw/litter	46.06	48.44	46.05	42.75
% Resorbed conceptuses/litter	7.7±16	6.3±9.8	3.5±8	13.5±17.2
N° litters evaluated	16	16	19	11
	Skeletal alterations: litter/fetal incidence n° (%)			
Hyoid, Alae, angulated	1/1 (6.2/0.9%)	2/2 (12.5/1.9)	2/2 (10.5/1.4)	3/3 (27%/4%)
	Historical data from laboratory			
Hyoid, Alae, angulated (See final table below for a breakdown of studies from which it was derived)	Litter incidence 0 – 35% Fetal incidence 0 – 5.3%			

[a]One rabbit was sacrificed moribund on day 18 of gestation prior to sacrifice, this rabbit was observed to have corneal opacity, lacrimation, ataxia, decreased motor activity, increased sensitivity to touch in the abdominal area and a lack of muscular control in the hindlegs.
[b]ulcerations in cardiac, pyloric and/or fundic regions
/ Rabbits / days
** Significantly different from vehicle control value, at P≤0.01.
↓ Statistically significantly different from control at at P≤0.05; () not significantly different from control

Table 3.10.1-1b: Uterine contents and litter data in individual rabbits which died, were sacrificed moribund, aborted or delivered naturally

Dose Group (mg/kg bw/day animal number)	Day of termination or death	Corpora lutea			Implantations			Embryos or fetuses[a]				Resorptions[b]			
		R	L	T	R	L	T	R	L	A/Del	T	R	L	A/Del	T
0 (vehicle) 10409	Delivered and sacrificed on day 28 of gestation	6	5	11	2	4	6	0	1[c]	2[c]	3[c]	1(LR)	1(LR)	1(LR)	3(LR)
20 10452	Delivered and sacrificed on day 29 of gestation	5	5	10	5	5	10	3[d]	3[d]	4[d]	10[d]	0	0	0	0
200 10461	Aborted and sacrificed on day 26 of gestation	5	6	11	4	5	9	0	0	8[e]	8[e]	0	0	-	-[e]
200 10463	Moribund sacrificed on day 18 of presumed gestation	6	5	11	0	0	0	0	0	-	0	0	0	-	0
200 10464	Aborted and sacrificed on day 24 of gestation	4	7	11	4	5	9	0	0	-[f]	-[f]	0	0	6(LR)[f]	-[f]
200 10469	Aborted and sacrificed on day 24 of gestation	5	5	10	1	0	1	0	0	1[g]	1[g]	0	0	0	0
200 10472	Found dead on day 14 of gestation	5	4	9	5	4	9	0	0	-	0	5	4	-	9
200 10480	Found dead on day 20 of gestation	4	7	11	4	7	11	3	5	-	8[b]	1(LR)	2(LR)	-	3(LR)

R Right; L Left; A Aborted; T Total; LR Late resorption

[a] Live unless noted otherwise

[b] Early unless noted otherwise

[c] Two delivered pups and one late resorption were found in the cage pan. One fetus was found in utero. All conceptuses appeared to have been alive at the time of delivery and normal for their developmental ages.

[d] Four delivered pups and one placenta were found in the cage pan. One delivered pup was observed to have a cannibalized tail. All remaining fetuses and delivered pups appeared to have been alive at the time of delivery and normal for their developmental ages.

[e] Eight aborted fetuses (four with placentas attached) and five placentas were found in the cage pan. Aborted fetuses appeared to have been alive at the time of abortion and normal for their developmental ages. Remaining conceptus was presumed to have been cannibalized.

[f] Six late resorptions were found in the cage pan. Remaining conceptuses were presumed to have been cannibalized.

[g] One aborted fetus was found in the cage pan. Aborted fetus appeared to have been alive at the time of abortion and normal for its developmental age.

[h] Fetuses found in utero appeared to have been alive at the time of maternal death and normal for their developmental ages.

Historical control data for New Zealand White Rabbits sourced from Hazleton Research Animals, Denver, Pennsylvania, USA and used at **Argus Research Laboratories**, Pensylvania, USA

Table 3.10.1-2c: Data is from studies conducted within 2.5 year of the conduct of Anonymous (1986)

Study Code	**1**	**2**	**6**	**7**	**10**	**12**	**13**	**14**	**16**	**17**	**18**	**23**	**24**	**25**	**26**	**27**			
Study type	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II	Seg II			
Vehicle	Propanol/ water	0.5% CMC	3.0% cornstarch suspension	3.0% cornstarch suspension	0.25% CMC	None - sham	Water	Water	Tween80 and CMC	Water	Water	0.5% CMC	0.5 hydroxypropyl cellose	Not known	0.5% Tween and 0.7% CMC	Corn oil			
Dose vol (mL/Kg))	2	5	10	10	10/5	None - sham	10	0.75	5	Drinking water	5	5	5	0.83	5	2			
Route	Topical	Oral	Oral	Oral	Oral	Intra uterine implant	Oral	IV	Oral	Drinking water	Oral	Oral	Oral	I.V.	Oral	Oral			
First date of C-section	31/07/1984	31/01/1984	26/06/1984	09/10/1984	07/11/1983	21/05/1984	31/03/1984	21/08/1984	13/09/1983	17/04/1984	15/08/1983	26/02/1985	17/06/1985	22/02/1985	03/12/1984	23/04/1985			
Litters	20	20	12	17	12	15	17	17	16	15	16	15	20	19	18	14			
Fetuses	138	97	98	112	87	90	150	110	96	99	133	105	135	135	111	101			
Hyoid Angulated																	**mean**	**Min**	**Max**
Litters	0	0	0	6	0	2	0	2	0	0	3	3	1	5	3	3			
%	0	0	0	35	0	13	0	12	0	0	19	20	5	26	17	21	**10.6**	**0**	**35**
Fetuses	0	0	0	6	0	2	0	2	0	0	3	3	1	6	3	3			
%	0	0	0	5.3	0	2.2	0	1.8	0	0	2.2	2.8	0.7	4.4	2.6	3	**1.6**	**0**	**5.3**

Maternal data: Deaths at top dose were compound related; 1 moribund sacrificed rabbit was not pregnant. Clinical signs of corneal opacity, lacrimation, ataxia, decreased motor activity, loss of hindlimb voluntary muscle control and increased sensitivity to touch in the abdominal region occurred for the doe that was sacrificed due to moribund condition. These effects were considered compound related. In the two other rabbits, dried faeces, decreased food consumption, weight loss and gastric ulceration occurred. These effects were considered to be compound related. Gastric ulceration also occurred for some surviving does given the 200 mg/kg bw/day. Abortion was seen at top dose.

At 200 mg/kg bw/day the average body weight gains were inhibited for days 6 - 12 of gestation. Food consumption was slightly decreased without reaching statistical significance. Gastric ulceration occurred for the top dose rabbits, 4 of the does with gastric ulceration died, were moribund sacrificed or aborted. At necropsy a high dose group rabbit had light brown kidneys, pale brown liver and red-brown coloured urine.

At the high dose the average number of resorptions and the average number of resorbed conceptuses per litter was slightly increased. Since these changes were not statistically significant and did not result in a decrease of the average litter size for the high dose group, this was not regarded as direct adverse effect, but secondary to the high toxicity to dams.

Litter data: Number of corpora lutea, implantations, live litter sizes or average foetal sex ratios were not altered. There was a small reduction in average foetal body weight per litter for the high dosage group without reaching statistical significance, which is regarded as a secondary consequence of the high maternal toxicity, but not as a primary effect. No foetal gross external and soft tissue alterations were observed in this study. No altered average number of foetal ossification sites that were attributable to the administration of Bifenox were seen.

A slightly increased incidence of angulated hyoid alae was reported at the top dose. The finding hyoid, alae, angulated will be discussed in more detail. The incidences of this finding are summarized in the following table.

Table 3.10.1-2 Selected findings in the rabbit developmental toxicity study

Dose (mg/kg bw)	**0**	**2**	**20**	**200**
Litters evaluated	16	16	19	11
Fetuses evaluated	106	106	143	75
Hyoid alae, angulated				
Number of litter (%)	1 (6.2)	2 (12.5)	2 (10.5)	3 (27.3)
Number of fetuses (%)	1 (0.9)	2 (1.9)	2 (1.4)	3 (4.0)
Historical control data (HCD)				
Number of litters (%) Number of foetuses (%)	29 (8.63) 32 (1.29)			

It can be seen that foetal incidence of hyoid angulated alae is increased at the highest dose. However, the foetal and litter incidences of the control group are at the upper end of the Historical Control Data (HCD) range, which indicates that this batch of animals apparently had already a normal higher genetic background for this finding which in this case can be regarded as normal for these animals. Therefore, it is questionable that the foetal and litter increases indicate a treatment effect, since for these animals a higher background rate can be assumed. A treatment relationship has to be questioned. Furthermore, the highest dose was maternal-toxic as evidenced by the death and occurrence of moribund animals and gastric ulceration at that dose. Especially the gastric ulceration indicates that the animals were under severe stress so that in principle this dose should not be used for evaluation of developmental endpoints. Overall therefore, a relevance of this finding for human safety is not seen.

The NOAEL for maternal toxicity was set at 20 mg/kg bw/day, based on mortality, clinical signs, reduced body weight gain and food consumption observed at 200 mg/kg bw/day.

The NOAEL for developmental toxicity was set at 20 mg/kg bw/day, taking into account the slightly increased incidence of hyoid alae angulated at 200 mg/kg bw/day.

3.10.1.4 Study 4 - CA 5.6.2 Developmental toxicity

Study reference

Report:	Anonymous (1986)
Title:	Rabbit teratology study Bifenox technical revised final report
Document No:	656-125, 656-125
Guidelines:	OECD 414
Deviations	None
GLP	Yes

Detailed study summary and results

Test substance, Test animals, Administration/exposure

Bifenox (batch # 3123142024, purity 97 %) was administered to 16 New Zealand White rabbits per dose level by stomach tube once daily on days 6 through 19 of gestation at dose levels of 5, 50, 160, 500 or 1000 mg/kg/day. Observations were made on clinical signs, mortality, body weight gain and feed consumption. On day 29 of presumed gestation, all surviving pregnant does were sacrificed and necropsied. The uterus from each female was excised, weighed, and examined for the number and placement of uterine implantation sites, number of live and dead foetuses, number of early and late resorbing foetuses, and abnormalities. Each foetus was examined to identify gender and external, visceral and skeletal alterations.

Results

Administration of 1000 mg/kg/day of Bifenox technical to New Zealand White rabbits resulted in the death of all high dose group rabbits. In the 500 mg/kg dose group, only one animal survived. The 500 and 1000 mg/kg dose group were excluded from evaluation. Seven animals were sacrificed after signs of abortion were noted. Pre-death clinical signs included hypoactivity, pale appearance, ataxia, and tremors.

Substantial mean body weight loss during treatment was recorded for both high-dose groups. A slight mean body weight loss was noted in animals dosed at 160 mg/kg bw/day over the last few days of the treatment period. This fluctuation in body weight was not significant when compared to the controls. Food consumption was greatly reduced in animals dosed at 500 and 1000 mg/kg bw/day during treatment. Food consumption in a1l other groups was similar to controls.

Table 3.10.1-3 Summary of findings (maternal and fetal) from the rabbit developmental toxicity study (Anonymous 1986)

Dose level [ppm]	**0**	**5**	**50**	**160**	**500**	**1000**
Mortality	0/16	0/16	1/16	0/16	14/16	16/16
Abortion or signs of imminent abortion	1	3	0	2	1	Group died
	Clinical signs					
Soft faeces:	0	1	0	0	1	
Hypoactive:	0	0	0	4	13	10
Slightly hypoactive:	0	0	0	2	15	14
Thin:	0	0	0	1	5	1
Ashen or pale appearance:	0	0	0	1	9	4
Ataxia:	0	0	0	1	2	3
Tremors:	0	0	0	0	1	6
	Body weight					
Body weight (day 6) [g]	4260±462	4017±358	4139±350	4078±331	4110±375	4063±356
Body weight (day 11) [g]	4273±442	4064±332	4201±357	4141±319	3667±431	3379±374b

Body weight (final) [g]	4401±443	4192±304	4305±414	4144±363	4246a	Group died
Body weight gain [g]	281 ± 248	311 ± 134	284 ± 243	229 ± 282 (-19%)		
Food consumption (G.-days 20-24) [mg]	580 + 347	479 + 239 (-18%)	495 + 260 (-15%)	410 + 321 (-30%)		
	Reproductive indices and fetal paramaters					
Corpora lutea[b] / dam	12	11	13	12	16	
Implantations[b] / dam	6	7	6	8	12	
Implantation efficiency	53.0	71.0	66.3	71.7	75.0	
Mean early resorptions (%)	1 (19.9)	1 (22.3)	1 (10.6)	2 (16.3)	0 (0.0)	
Mean late resorptions (%)	0 (0.0)	0 (0.0)	0 (5.2)	0 (0.7)	0 (0.0)	
Mean total resorptions (%)	1 (19.9)	1 (22.3)	1 (15.8)	2 (17.0)	0 (0.0)	
Mean number dead foetuses (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Mean number live foetuses (%)	5 (80.1)	6 (77.8)	7 (84.2)	7 (83.0)	12 (100.0)	
Mean number of litters with viable foetuses	11	9	13	11	1	
Mean number of male foetuses (%)	3 (56.1)	3 (41.4)	4 (43.7)	4 (46.8)	7 (58.3)	
Mean viable fetal weights (g)	46.7	45.5	41.5	40.6	35.1	
Males	47.8	45.3	42.2	43.1	33.4	
Females	45.0	44.5	41.1	39.5	37.5	
	Malformations and variations					
	Fetal external malformations					
Fetuses evaluated N	59	62	96	82	12	
Live N	59	62	96	82	12	
Dead N	0	0	0	0	0	
Short tail						
Fetal incidence N(%)	0	0	1 (1.0)	0	0	
Litter incidence N(%)	0	0	1 (7.7	0	0	
	Hyperflexion forepaw (both)					
Fetal incidence N(%)	0	0	1 (1.0)	0	0	
Litter incidence N(%)	0	0	1 (7.7	0	0	
	Midline closure effect[c]					
Fetal incidence N(%)	0	0	0	1(1.2)	0	
Litter incidence N(%)	0	0	0	1(9.1)	0	
	Fetal visceral variations					
Litters evaluated N	11	9	13	11	1	
Fetuses evaluated (total)[d] N	59	62	96	82	12	
Fetuses evaluated (intact)[d] N	33	35	52	44		
Live N	59	62	96	82	12	
Dead N	0	0	0	0	0	
	Dark area, right side, maxilla, approximately 4 mm in length[d]					
Fetal incidence N(%)	0	0	1 (1.0)	0	0	
Litter incidence N(%)	0	0	1(7.7)	0	0	
	Left carotid arising from innominate artery					
Fetal incidence N(%)	0	0	1 (1.0)	0	1 (8.3)	
Litter incidence N(%)	0	0	1 (7.7)	0	1 (100.0)	

	Lung – intermediate lobe agenesis					
Fetal incidence N(%)	11 (18.6)	7 (11.3)	6 (6.3)	8 (9.8)	1 (8.3)	
Litter incidence N(%)	3 (27.7)	4 (44.4)	3 (23.1)	4 (36.4)	1 (100.0)	
	Lung – intermediate lobe hypoplastic					
Fetal incidence N(%)	0	1 (1.6)	1 (1.0)	0	0	
Litter incidence N(%)	0	1 (11.1)	1 (7.7)	0	0	
	Fetal visceral malformations					
Litters evaluated N	11	9	13	11	1	
Fetuses evaluated N	59	62	96	82	12	
Live N	59	62	96	82	12	
Dead N	0	0	0	0	0	
	Heart and/or great vessel anomaly[e]					
Fetal incidence N(%)	0	0	2 (2.1)	0	0	
Litter incidence N(%)	0	0	1 (7.7)	0	0	
	Midline closure effect[c] (also in external malformations above)					
Fetal incidence N(%)	0	0	0	1 (1.2)	0	
Litter incidence N(%)	0	0	0	1 (9.1)	0	
	Fetal skeletal variations					
Litters evaluated N	11	9	13	11	1	
Fetuses evaluated (total)[d] N	59	62	96	82	12	
Fetuses evaluated (intact)[d] N	33	35	52	44		
Live N	59	62	96	82	12	
Dead N	0	0	0	0	0	
	Skull-accessory bone					
Fetal incidence N(%)	1 (3.0)	0	2 (3.9)	1 (2.3)	0	
Litter incidence N(%)	1(9.1)	0	2 (15.4)	1 (9.1)	0	
	Frontal – incompletely					
Fetal incidence N(%)	0	0	2 (3.9)	0	0	
Litter incidence N(%)	0	0	1 (7.7)	0	0	
	Interparietal – incompletely ossified					
Fetal incidence N(%)	0	0	1 (1.9)	0	0	
Litter incidence N(%)	0	0	1 (7.7)	0	0	
	Thoracic vertebral centra misaligned					
Fetal incidence N(%)	0	1 (1.6)	0	0	0	
Litter incidence N(%)	0	1 (11.1)	0	0	0	
	Lumbar arches interrupted ossification					
Fetal incidence N(%)	0	0	1 (1.0)	1 (1.2)	0	
Litter incidence N(%)	0	0	1 (7.7)	1 (9.1)	0	
	Caudal vertebrae – misaligned					
Fetal incidence N(%)	0	0	1 (1.0)	0	0	
Litter incidence N(%)	0	0	1 (7.7)	0	0	
	26 presacral vertebra count					
Fetal incidence N(%)	3 (5.1)	6 (9.7)	8 (8.3)	8 (9.8)	0	
Litter incidence N(%)	3 (27.3)	2 (22.2)	6 (46.2)	5 (45.5)	0	

	Centra bipartites					
Fetal incidence N(%)	0	0	1 (1.0)	0	0	
Litter incidence N(%)	0	0	1 (7.7)	0	0	
	Sternebra 5th not ossified					
Fetal incidence N(%)	9 (15.3)	13 (21.0)	10 (10.4)	12 (14.6)	1 (8.3)	
Litter incidence N(%)	6 (54.5)	5 (55.6)	6 (46.2)	6 (54.5)	1 (100.0)	
	Sternebra 5th bipartite					
Fetal incidence N(%)	1 (1.7)	0	1 (1.0)	0	0	
Litter incidence N(%)	1 (9.1)	0	1 (7.7)	0	0	
	Sternebra 6th bipartite					
Fetal incidence N(%)	1 (1.7)	0	2 (2.1)	2 (2.4)	0	
Litter incidence N(%)	1 (9.1)	0	2 (15.4)	2 (18.2)	0	
	Sternebra 6th not ossified					
Fetal incidence N(%)	1 (1.7)	1 (1.6)	4 (4.2)	5 (6.1)	0	
Litter incidence N(%)	1 (9.1)	1 (11.1)	3 (23.1)	4 (36.4)	0	
	Sternebra 2nd not ossified					
Fetal incidence N(%)	0	0	0	1 (1.2)	0	
Litter incidence N(%)	0	0	0	1 (9.1)	0	
	Sternebra 2nd bipartite					
Fetal incidence N(%)	0	0	0	1 (1.2)	0	
Litter incidence N(%)	0	0	0	1 (9.1)	0	
	Sternebrae Misaligned					
Fetal incidence N(%)	0	0	0	1 (1.2)	0	
Litter incidence N(%)	0	0	0	1 (9.1)	0	
	13th full rib –unilateral					
Fetal incidence N(%)	9 (15.3)	0	7 (7.3)	8 (9.8)	3 (25.0)	
Litter incidence N(%)	6 (54.5)	0	6 (46.2)	5 (45.5)	1 (100.0)	
	13th Rudimentary rib – unilateral					
Fetal incidence N(%)	12 (20.3)	6 (9.7)	22 (22.9)	11.8(13.4)	1 (8.3)	
Litter incidence N(%)	7 (63.6)	5 (55.6)	9 69.2()	8 (72.7)	1 (100.0)	
	13th full ribs –bilateral					
Fetal incidence N(%)	10 (16.9)	12 (19.4)	29 (30.2)	20 (24.4)	1 (8.3)	
Litter incidence N(%)	7 (63.6)	5 (55.6)	12 (92.3)	8 (72.7)	1 (100.0)	
	Ribs 13th Floating					
Fetal incidence N(%)	3 (5.1)	3 (4.8)	1 (1.0)	5 (6.1)	0	
Litter incidence N(%)	2 (18.2)	2 (22.2)	1 (7.7)	4 (36.4)	0	
	13 Rudimentary ribs - bilateral					
Fetal incidence N(%)	1 (1.7)	1 (1.6)	6 (6.3)	8 (9.8)	1 (8.3)	
Litter incidence N(%)	1 (9.1)	1 (11.1)	5 (38.5)	6 (54.4)	1 (100.0)	
	Ribs - forked					
Fetal incidence N(%)	1 (1.6)	1 (1.0)	1 (1.0)	1 (1.2)	0	
Litter incidence N(%)	1 (11.1)	1 (7.7)	1 (7.7)	1 (9.1)	0	
	Ribs – interrupted ossification					
Fetal incidence N(%)	0	0	0	1 (1.2)	0	
Litter incidence N(%)	0	0	0	1 (9.1)	0	
	Rib(s) – extra					
Fetal incidence N(%)	0	0	0	1 (1.2)	0	

Litter incidence N(%)	0	0	0	1 (9.1)	0	
	First thoracic rib(s) small					
Fetal incidence N(%)	0	0	1 (1.0)	0	0	
Litter incidence N(%)	0	0	1 (7.7)	0	0	
	Metacarpals and phalanges less than 19 count					
Fetal incidence N(%)	0	0	1 (1.0)	0	0	
Litter incidence N(%)	0	0	1 (7.7)	0	0	
	Metacarpals and phalanges misaligned					
Fetal incidence N(%)	0	0	0	0	2 (16.7)	
Litter incidence N(%)	0	0	0	0	1 (100.0)	
	Skeletal malformations					
	Centra fused					
Fetal incidence N(%)	0	0	2 (2.1)	0	0	
Litter incidence N(%)	0	0	1 (7.7)	0	0	
	Vertebral anomaly with associated rib anomaly					
Fetal incidence N(%)	0	0	1 (1.0)	0	0	
Litter incidence N(%)	0	0	1 (7.7)	0	0	
	Stenebrae fused					
Fetal incidence N(%)	0	0	0	1 (1.2)	0	
Litter incidence N(%)	0	0	0	1 (9.1)	0	
	Ribs fused					
Fetal incidence N(%)	1 (1.7)	0	0	0	0	
Litter incidence N(%)	1 (9.1)	0	0	0	0	
	Summary of External, visceral and skeletal findings by fetus					
Fetuses evaluated N	59	62	96	82	12	
Fetuses with any malformation N (%)	1 (1.7)	0	6 (6.3)	2 (2.4)	0	
Litters evaluated N	11	9	13	11	1	
Litters with any malformation N	1	0	3	2	0	
Percent with any malformation	9.0	0	23.1	18.2	0	
	Total findings by litter					
	External					
Litters with variants N (%)	0	0	0	0	0	
Litters with malformations N (%)	0	0	2 (15.4)	1 (9.1)	0	
	Visceral					
Litters with variants N (%)	3 (21.3)	4 (44.4)	5 (38.5)	4 (36.4)	1 (100.0)	
Litters with malformations N (%)	0	0	1 (7.7)	1 (9.1)	0	
	Skeletal					
Litters with variants N (%)	11 (100.0)	9 (100.0)	13 (100.0)	11 (100.0)	1 (100.0)	
Litters with malformations N (%)	1 (9.1)	0	1 (7.7)	2 (18.2)	0	

[a] Only one survivor by this point
[b] all animals started losing bodyweight from the beginning of dosing and were lost between day 8 and 24
[c] liver protruding through umbilicus
[d] Fetuses evaluated (Total) includes all foetuses, those without heads and those with heads (foetuses evaluated intact); whereas, foetuses evaluated (intact) include only foetuses with heads.
[e] one dextrocardia, one major heart anomaly.

Mean number of corpora lutea and implantations as well as foetal viability and foetal sex distribution were comparable for all groups. Mean foetal body weights decreased across groups in a dose-related, but not statistically significant manner. Foetal skeletal and visceral variations were noted in all groups with foetuses available for examination. The incidence was not dose related. A single malformation was observed in the control group. Six foetuses in three litters at 50 mg/kg bw/day had malformations and two foetuses in two litters at 160 mg/kg bw/day. The single litter available for evaluation at 500 mg/kg bw/day had no malformations.

Signs of slight maternal toxicity were noted at 160 mg/kg bw/day. At 50 mg/kg bw/day, one female was found dead during the treatment period (prior to dosing on Day 15). No other evidence of maternal toxicity was observed. No abortions occurred in this group. Body weights and food consumption were comparable to the control values. Foetal malformations in this group included hyperflexed paws, two heart anomalies (in one litter), and one litter containing foetuses with fused vertebral centra and one foetus with multiple anomalies including the vertebral column.

Marked maternal toxicity resulted from doses at and above 500 mg/kg/day and included increased incidences of death, abortion, and reduced body weight gain and food consumption.

The incidence of spontaneous abortions did not occur in a dose-related pattern; therefore, it is not clear whether the three abortions are attributable to treatment with Bifenox technical. Except for these three abortions, females had no observable signs of adult or foetal toxicity or teratogenicity at 160 mg/kg bw/day. Foetal malformation did not occur in a dose-related pattern.

No increased incidence of the variation (hyoid alae angulated) noted in the Anonymous (1986) study was noted.

On the basis of these observations, the maternal no-effect level (NOEL) was proposed to be 50 mg/kg/day.

However, without any effects noted on foetuses it is considered that the developmental NOAEL is 160 mg/kg bw/day.

3.10.2 Human data

No human data available on reproduction toxicity of Bifenox.

3.10.3 Other data (e.g. studies on mechanism of action)

No other data available on reproduction toxicity of Bifenox.

3.11 Specific target organ toxicity – single exposure

3.11.1 Animal data

Adverse effects, that were noted in acute toxicity studies in experimental animals included faecal staining, soft stool and hypoactivity. Those clinical findings were related to acute systemic toxicity due to a single exposure at very high dose levels and were fully reversible within the period of the experiment. Neither the adverse effects nor necropsy findings in those acute toxicity studies could be related to a specific target organ toxicity. Bifenox is of low toxicity.

3.11.2 Human data

No human data available on specific target organ toxicity of Bifenox. No clinical cases are reported. There are no observations in humans indicating any adverse effects upon a single dose of Bifenox. Bifenox is of low toxicity.

3.11.3 Other data

No other data available on specific target organ toxicity of Bifenox.

3.12 Specific target organ toxicity – repeated exposure

3.12.1 Animal data

3.12.1.1 Study 1 – CA 5.3.2 Oral 90 day studies

Study reference

Report:	Anonymous (1982)
Title:	13-Week dietary toxicity study in rats MCTR-299-79
Document No:	International Research and Development Corporation, 440132
Guidelines:	Not stated, the study was in accordance with OECD Guideline No. 408.
GLP	Yes

Detailed study summary and results:

This study does not provide a statement of GLP but the study report claims GLP compliance based on an attest of QAU. In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool)[1] has been used for evaluation. Consequently, this study is categorized into Klimisch[2] category 1: reliable without restriction. Therefore, it is concluded that this study is valid, and is considered for classification.

Test substance, Test animals, Administration/exposure

20 Charles River CD rats/sex/dose received Bifenox (batch # 01098001 of unknown purity) in the diet. The dose levels were at 0, 300, 900 and 2500 mg/kg bw/day. The diet was analysed for homogeneity of distribution and stability. Diet samples contained 84 - 111% of target concentration and reached 96-105% of the desired level.

Results

After 13-weeks dietary exposure, mortality was observed in a total of 11 animals from the highest dose group (2500 mg/kg bw), in 2 animals from the 900 mg/kg bw/day group, 1 in the lowest dose group and 1 animal from the control group (0 mg/kg bw/day) did not survive.

Clinical signs that occurred at the top dose were infrequent signs such as emaciation, brownish-red urine, red material in urine, pale skin and non-agonal coldness-to-the-touch. Additional signs noted for 3 to 6 treated rats were excessive lacrimation, scabbed areas on the body and subcutaneous neck masses. Prior to death or in extremis sacrifice, animals showed laboured breathing, black material around nose and eyes and coldness-to-the-touch.

The body weight was statistically decreased at 900 and 2500 mg/kg bw/day at week 1. Food consumption was decreased at 900 and 2500 mg/kg bw/day and paralleled the changes in mean body weight.

Some slight changes of haematology were seen at 900 and 2500 mg/kg bw/day. Slightly higher reticulocyte counts were noted at top dose, which could indicate a partial compensation for the slightly decreased haematological parameters noted.

Changes in clinical chemistry were noted in increased serum alkaline phosphatase in male and female rats at 900 and 2500 mg/kg bw/day. Cholesterol was increased for females at top dose and total protein and globulin were decreased in

[1] The ToxRTool is the outcome of a project sponsored by the European Commission (CCR.IHCP.C433199.XO). The ToxRTool is property of the European Commission: © European Communities

[2] Klimisch et al., 1997, Regulatory Toxicology Pharmacology 25(1), 1-5

male rats only at the two top doses. Some of these parameters could suggest liver toxicity. Creatinine was increased in male rats.

Urinanalysis showed a slight reduction in specific gravity in top dose female rats. Elevated urobilinogen levels ranging from slight for rats at the low dose, slight to moderate for rat at intermediate dose and from slight to marked for rats at top dose were observed. Urobilinogen is formed in the small intestine and the colon. A very small amount is therefore excreted into the urine and this amount will increase when more urobilinogen is formed or when the liver is sick and unable to re-excrete it.

Organ weight analysis showed a significant decrease in ovarian weight of females receiving the different doses. The ovaries were free of any microscopically detectable changes and no dose-response relationship was apparent. There were statistically significant increases in relative liver weight among male and female rat at 900 and 2500 mg/kg bw/day. Relative weight of testis was increased at the two top doses. Most of the changes were considered due to the reduction in body weight and were not associated with histo-pathological findings. Macroscopic observation did not indicate any test-article related effects.

Microscopic evaluation showed pyelonephritis in several animals from the high dose group. The kidneys had a suppurative infection and this pathology was diagnosed as the cause of death in 8 of the treated animals, which died during the study. Pyelonephritis is a rare finding in control rats. There was no indication in the clinical chemistry or urinanalysis data of kidney pathology. The pyelonephritis and/or renal or urinary calculi observed in treated rats would induce the significant ante mortem signs of emaciation, coloured urine and red material in urine.

The macroscopic and microscopic examinations permit the speculation that the test article may have precipitated out in the kidney or urinary bladder causing obstruction to renal flow, which subsequently led to infection. The significance of the single case in a 300 mg/kg bw/day female was considered equivocal.

Table 3.12.1-1 Endpoints from 13 week feeding study in rats with Bifenox

Endpoints	0 [mg/kg bw/day]		300 [mg/kg bw/day]		900 [mg/kg bw/day]		2500 [mg/kg bw/day]	
	Male	Female	Male	Female	Male	Female	Male	Female
Compound intake [mg/kg bw/day]			292	319	882	960	2478	2697
Mortality	1	0	1	0	0	2	4	7
Clinical signs	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Please refer to text	
Body weight	n.a.	n.a.	n.a.	n.a.	↓ 16.0%	↓ 7.7%	↓ 29.8%	↓ 22.0%
Food consumption	n.a.	n.a.	↓ 1.5%	n.a.	↓ 12.6%	↓ 6.6%	28.3%	24.6%
Haematology								
Hb	n.a.	n.a.	n.a.	n.a.	↓ 3%	↓ 5%	↓ 13%	↓ 10%
Ht	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 11%	↓ 8%
RBCs	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 12%	↓ 6%
Reticulocytes week 6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	(↑ 21%)	↑ 60%
Reticulocytes week 13	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	(↑ 48%)	↑ 34%
Clinical chemistry								
AP	n.a.	n.a.	n.a.	n.a.	↑ 60%	↑ 26%	↑ 100%	↑ 105%
Total protein	n.a.	n.a.	n.a.	n.a.	↓ 8%	n.a.	↓18%	n.a.
Creatinine	n.a.	n.a.	n.a.	n.a.	↑ 16%	n.a.	↑ 33%	n.a.
Globulin	n.a.	n.a.	n.a.	n.a.	↓ 18%	n.a.	↓ 28%	n.a.
Cholesterol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑ 73%
Total bilirubin mg/dL week 6	0.2	0.2	0.1	0.1*	0.1*	0.1*	0.1	0.1*
Urinalysis								
Specific gravity	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 1.5%
pH	9	6	8*	6	7*	7	7*	7
Urobilinogen (EU/dL)	0.1	0.1	1	0.1	1-4	1-4	4-8	0.1-8
Gross pathology								
Calculi/gritty material pelvis	0	2/20	1/20	0/20	0	2/20	0	2/20
Hydronephrosis/dilated pelvis	0	1/20	1/20	1/20	1/20	0	0	0
Urinary bladder calculi	0	2/20	0	1/20	0	2/20	1/20	0
Histopathology: number affected rats / number of rats examined								
Pyelonephritis	0	0	0	1**	0	1/20	6/20	7/20
Liver necrosis	0	1/20	2/20	0	1/20	0	1/20	1/20
Relative organ weight								
Ovary / testis	n.a.	n.a.	n.a.	↓ 35%	↑ 17%	↓ 25%	↑ 42%	↓ 17%
Liver	n.a.	n.a.	n.a.	n.a.	↑ 20%	↑ 16%	↑ 50%	↑ 61%
Kidney	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑ 23%
Brain	n.a.	n.a.	n.a.	n.a.	↑ 17%	↓ 8%	↑ 45%	↓ 25%

↓, ↑, * statistically significant from control
( ) not statistically significant
** single case at terminal sacrifice, considered equivocal
n.a. no data available

Conclusion

The high dose and the middle dose were considered toxic in this study, causing death, loss of body weight and renal lesions. Slight hematotoxicity was seen at the top dose. The NOAEL was determined to be 300 mg/kg bw/day, not taking into account the single pyelonephritis observed at this dose level in females rats.

3.12.1.2 Study 2 – CA 5.3.2 Oral 90 day studies

Study reference

Report:	Anonymous (1986)
Title:	Bifenox oral toxicity study in beagle dogs (repeated daily dosage for 52 weeks)
Document No:	RNP 218/85998
Guidelines:	Not stated, the study was in accordance with OECD Guideline No. 409 and EC Directive 87/302 part B
GLP	Yes

Detailed study summary and results

This study does not provide a statement of GLP but the study report claims GLP compliance. In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool)[1] has been used for evaluation. Consequently, this study is categorized into Klimisch[2] category 1: reliable without restriction. Therefore, it is concluded that this study is valid and is considered for classification.

Test substance, Test animals, Administration/exposure

Six Beagle dogs/sex/dose received a gelatine capsule of Bifenox (lot No. 353-12-1, purity: 98%) at 20, 145 or 1000 mg/kg bw/day for 52-weeks of administration. After 26 weeks, 2 male and 2 female dogs were killed from each dose group.

Statistical analysis: Barlett's test was applied to test heterogeneity of variance between treatments. If a significant heterogeneity was detected, an analysis of variance was carried out. If no satisfactory transformation was found, the Kruskal-Wallis analysis of ranks was used. Analysis of variance was followed by Student's "t" test and Williams test for a dose related response. The Kruskal-Wallis analyses were followed by the non-parametric equivalents of the t test and Williams test. Organ weight was analysed by analysis of variance or covariance.

Results

In this study, the MTD was not reached.

Oral administration of Bifenox for 52 weeks at 1000 mg/kg bw/day did not elicit signs related to treatment and no animals died. Body weight, body weight gain and food consumption were not affected by treatment. Ophthalmoscopic examinations and urinanalysis revealed no changes that were attributed to treatment. Marrow smears were found to be normal.

The haematology shows only slightly reduced red blood cell parameters during week 26 in male dogs receiving 1000 mg/kg bw/day.

Clinical chemistry during week 13, 26 and 52 revealed mean values for serum calcium for male dogs at top dose that were lower than control values. A similar pattern was not apparent for the female dogs and the toxicological importance of this finding is uncertain. During week 52, at top dose, male dogs had higher GPT and OCT than control. Serum ornithine carbamoyltransferase (SOCT) is a diagnostic marker of hepatic disorders due to its localization in periportal mitochondria. Similar findings were not recorded for females.

The analysis of organ weights showed an alteration in kidney weights for dogs receiving 1000 mg/kg bw/day at week 26. After 52 weeks, relative liver and kidney weights were significantly higher at top dose for male and female dogs. Ovary weights were increased.

Histopathological examination at week 26 reported foci of cortical basophilic tubules in kidneys of dogs at 145 mg/kg bw/day and 1000 mg/kg bw/day. These changes were considered fortuitous. At week 52, no abnormal histological

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[2] Klimisch et al., 1997, Regulatory Toxicology Pharmacology 25(1), 1-5

findings were seen except for 1 liver fibrosis in 1 female dog at top dose. Changes consistent with estrus, met estrus and anestrus were seen in uterus, ovaries and vagina of some dogs, which were not considered to be compound related.

Table 3.12.1-2 Results from 1-year feeding study in dogs with Bifenox

Endpoints	0 [mg/kg bw/day]		20 [mg/kg bw/day]		145 [mg/kg bw/day]		1000 [mg/kg bw/day]	
	Male	Female	Male	Female	Male	Female	Male	Female
Week 26								
Haematology								
Hb	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 10%	n.a.
RBCs	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 15%	n.a.
MCV	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑ 6%	n.a.
Platelets	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑ 28%	n.a.
Clinical chemistry								
Albumin	n.a.	n.a.	n.a.	n.a.	↓ 10%	n.a.	↓ 13%	↓ 10%
Urea	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 19%	↓ 19%	↓ 27%
Creatinine	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 23%	n.a.
AP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 42%	n.a.
Ca^{2+}	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 7%	n.a.
Organ weight absolute								
Kidneys	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑ 30%
Liver	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	(↑ 23%)
Spleen	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	(↑ 16%)
Thymus	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	(↑ 32%)
Histopathology: number affected dogs / number examined								
Kidney: foci of cortical basophilic tubules	n.a.	n.a.	n.a.	n.a.	1/2	2/2	2/2	n.a.
Week 52								
Clinical chemistry								
GPt	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑ 75%	n.a.
OCT	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑ 83%	n.a.
Ca^{2+}	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 10%	n.a.
P	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 14%	n.a.
Cl^-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↓ 3%	n.a.
Organ weights relative								
Liver	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑ 38%	↑ 23%
Kidneys	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	↑ 46%	↑ 22%
Ovaries	n.a.	n.a.	n.a.	(↑ 36%)	n.a.	↑ 51%	n.a.	↑ 57%
Histopathology: number affected dogs / number examined								
Liver fibrosis	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1/4

↓↑* statistically significant from control
( ) not statistically significant
n.a. no data available

Conclusion

The NOAEL was set at 145 mg/kg bw/day due to increased OCT and GPT values after 52 week exposure seen at 1000 mg/kg bw/day. Slight blood toxicity at interim sacrifice was seen at top dose. Increased ovary weight was not taken into account: this effect was not associated with abnormal histopathological findings.

3.12.1.3 Study 2 – CA 5.3.2 Subchronic toxicity study in mice (90 days) at 200, 500,1000 ppm, bifenox (Anonymous 1979)

Mortality: no mortality was reported.

Clinical signs: no compound-related signs were seen.

Body weight increase was recorded for the high dose males and low dose males for weeks 3, 4, 5, 6, and 10, 11 12 respectively. These changes, however, were inconsistent and at all intervals the control and treated groups did not vary by more than 10%. The variations were judged to be toxicologically unimportant.

Food consumption increased for males treated at 500-ppm and for females treated with 1000-ppm for week 2 and 3, respectively. These were not judged to represent an adverse effect.

Necropsy findings: Sporadic mottling of lungs was seen which was interpreted as patchy congestion/hemorrhage resulting from the kill.

Organ weight: a dose-related increase in liver weight was seen in all exposed male mice and in top dose female mice.

Histopathology: scattered foci of proximal tubular vacuolization in the kidneys of all control males. The significance of this finding is unknown.

In any affected liver, the change was a diffuse hepatocellular hypertrophy, was panlobular and appeared usually as a slightly enlarged cytoplasmic compartment. The changes were minimal often equivocal and not easily quantifiable. They were more pronounced in males.

Although the specific ultrastructural basis for the changes was not clear from the material examined, they were compatible with hypertrophy of endoplasmic reticulum, an adaptative mechanism commonly associated with metabolism of drugs.

Table 3.12.1-3 13-week mice study

Endpoints/dose	**0**		**200 ppm**		**500 ppm**		**1000 ppm**	
Sex	**m**	**f**	**m**	**f**	**m**	**f**	**m**	**f**
Organ weight relative								
Liver			tl3%		18%T	T3%	29%t	T16%
Histopathology								
Hepatocellular hypertrophy, diffuse	0/10	0/10	0/10	0/10	0/10	0/10	10/10	3/10

T: Statistically significant modifications

Conclusion: survival, clinical signs, body weight and food intake suggested no compound-related adverse effects at any of the treatment levels. Hepatocellular hypertrophy revealed through histopathologic evaluation at 1000 ppm is suggestive of increased endoplasmic reticulum related to possible liver enzyme induction and is considered as an adaptative mechanism. NOAEL = 1000 ppm (115 mg/kg bw/d).

GLP status: no (only attest of QAU)

Guideline: study is not in compliance with dir EEC 87/302 part B or OECD test guideline 408.

Deviation from official protocol: hematological tests and clinical biochemistry tests not performed. Only liver, kidneys, testes and epididymes were weighed. Histopathology limited to liver and kidneys.

Material and methods: 25 mice B6C3F1 (Charles River breeding) strain/sex/dose received in their diet bifenox (lot n° 16230, 98.3%) at 200, 500 and 1000 ppm.

Statistical analyses: differences between the test group and control group were analysed using the Dunnett's t test. Due to the fact that the study is quite incomplete, the study is not suitable for final evaluation.

3.12.1.4 Study 3 – CA 5.3.3 other routes

Study reference

Report:	Anonymous (2002)
Title:	Bifenox: Twenty-eight day repeated dose dermal toxicity study in the rat
Document No:	Safepharm Laboratories Limited, 644/061
Guidelines:	EC Directive 92/69/EC and OECD 410
GLP	Yes

Detailed study summary and results:

In order to assess the current reliability of this study, the ToxRTool (Toxicological data reliability Assessment Tool)[1] has been used for evaluation. Consequently, this study is categorized into Klimisch[1] category 1: reliable without restriction. Therefore, it is concluded that this study is valid and is considered for classification.

Test substance, Test animals, Administration/exposure

5 rats Sprague-Dawley Crl: CD (SD) IGS BR se/dose were exposed to Bifenox (batch # 0546/1, purity 98.2%) suspended in arachis oil BP topically for 6 hours/day daily for 28 days onto shorn dorsal skin at 15, 150, 1000 mg/kg bw/day to 10% of the total body surface. After 6-hour exposure, skin was washed. Due to a technical error on day 2, animals 28, 29 and 30 were not decontaminated and consequently, were exposed for 24 hours. These animals were not dosed on day 3 after such an exposure. The site of application was semi-occluded.

Statistical analyses: haematological, blood chemistry and organ weight were analysed by one way analysis of variance incorporating Levene's test for homogeneity of variance. Pair wise comparisons were conducted using the Dunnett's test. For non-parametric methods, Kruskal Wallis ANOVA and Mann-Whitney U test.

Results

There were no deaths. No clinical observable signs of toxicity were detected. There were no dermal reactions seen that could be attributed to test material irritancy. At the top dose, during week 4, slight decreased body weight gain was seen in male and female animals (m: 27%; f: 22%); food consumption was slightly reduced in males (14%). These effects did not reach statistical significance. There were not treatment related changes in the haematological and blood chemistry parameters. A statistically significant increase in relative liver weight (13%) was seen at 1000 mg/kg bw/day for males. Necropsy findings did not show any abnormalities. A minimal focal hepatocyte necrosis was seen in one male at top dose.

Conclusion

Taking into account the minimal focal hepatocyte necrosis seen in one male and the decreased bw at 1000 mg/kg bw/day, the NOAEL = 150 mg/kg bw/day.

3.12.1.5 Evaluation of STOT-RE based on studies presented under 3.12.1

Animals repeatedly exposed to Bifenox in their diet developed mild signs of porphyria as suggested by small-altered blood parameters, kidney toxicity and some altered clinical chemistry, which could suggest hepatotoxicity Those effects are considered to be treatment related and occurred only at the highest dose levels of 900 mg/kg bw/day in rats (90 days) and 1000 mg/kg bw/day in dogs (90-days) or 47 mg/kg bw/day in mice (2 years).

Dermal repeated exposure revealed minor treatment related effects at the top dose (1000 mg/kg bw/d) that suggested hepatotoxicity are confirmed by systemic effects seen after oral repeated exposure.

[1] Klimisch et al., 1997, Regulatory Toxicology Pharmacology 25(1), 1-55

[2] The ToxRTool is the outcome of a project sponsored by the European Commission (CCR.IHCP.C433199.XO). The ToxRTool is property of the European Commission: © European Communities

Those effects occurred in all studies at the highest dose levels. Administration at lower dose levels did not exhibit any significant adverse effects due to the low toxicity of Bifenox.

3.12.2 Human data

No human data available on specific target organ toxicity of Bifenox after repeated exposure. No clinical cases are reporterted.

3.12.3 Other data

No other data available on specific target organ toxicity of Bifenox after repeated exposure.

3.13 Aspiration hazard

3.13.1 Animal data

Not relevant.

3.13.2 Human data

Not relevant.

3.13.3 Other data

Not relevant.

4 ENVIRONMENTAL HAZARDS

4.1 Degradation

4.1.1 Ready biodegradability (screening studies)

4.1.1.1 Study 1 – CA 7.2.2.1 Ready biodegradability

Study reference:

Report:	Anonymous (1989)
Title:	Study on the biodegradability of Bifenox according to modified sturm test (OECD guideline 301 B for testing chemicals and the 6th amendment of the council directive 67/548/EEC, 1984)
Document No:	440153, R 67 006 04
Guidelines:	OECD 301B
GLP	yes

Study summary and results:

In the study, a modified sturm test conducted according to OECD 301B, the ready biodegradability of Bifenox at a concentration of 10 and 20 mg a.s./L was investigated for up to 28 days at 23°C. Control and positive control vessels were also run. The positive control vessels fulfilled the validity criteria. After 28 days, cumulative CO_2 production by the mixtures containing Bifenox was 8.74 and 14.93 mg for the 10 and 20 mg/L test concentrations, respectively. This is equivalent to 14.0 and 11.8% of the theoretical amount of CO_2 that could be generated from the added test material. Bifenox can therefore be considered as not readily biodegradable.

4.1.2 BOD_5/COD

Please refer to point 4.1.1.

4.1.3 Aquatic simulation tests

4.1.3.1 Study 1 – CA 7.2.2.2 Aerobic mineralisation in surface water

Study reference:

Report:	KCA 7.2.2.2/01 - Anonymous (2015)
Title:	Aerobic mineralisation of [dichlorophenyl-ring-U-14C]Bifenox in surface water
Document No:	S14-03889, R-90017805
Guidelines:	OECD 309, 2004
GLP	yes

Study summary and results:

Aerobic mineralisation of [dichlorophenyl-U-^{14}C]Bifenox in surface water amended with sediment was investigated under laboratory conditions in the dark at two different concentrations. For this purpose, 5.16 µg/L and 49.3 µg/L of radiolabelled Bifenox was applied to 500 mL of natural lake water containing 0.5 g sediment. The test flasks were incubated for a period of up to 58 or 91 days, for the low and high test concentrations, respectively, at 20°C ± 2°C under

aerobic conditions with slight orbital movement. During this time, duplicate samples were taken for analysis at specified intervals on days 0, 1, 3, 5, 7, 14, 21, 28, 58 and 91 (high test concentration only).

Traps for organic volatiles (tenax tubes) and carbon dioxide (2M sodium hydroxide) were used. Radioactivity was quantified by LSC and characterised by normal phase TLC. Reversed phase TLC was used for confirmation of metabolites in selected samples.

Mean recoveries were within the range of 92.7%-110.1% AR for the low test concentration and 96.1%-105.8% AR for the high test concentration. Mineralisation was not extensive reaching means of 15.2% and 7.0% for the low and high concentrations, respectively, at the end of incubation. For both tested concentrations one major metabolite (M1 = bifenox acid) was formed with a mean maximum of 75.2% (low test concentration) after 14 days and 92.5% AR (high test concentration) after 28 days. Up to 6 minor unknown metabolites occurred with a mean maximum concentration of 4.2% AR. No individual unknown in a single replicate measured greater than 4.7%. No 2,4-Dichlorophenol and no Nitrofen was found in the samples by TLC. None of the "unknown" could be attributed to any of these compounds. By LC-MS measurement, the presence of bifenox acid as main product was confirmed by its retention time, accurate mass, isotopic pattern, the MS^2 product ion spectrum and the corresponding radiodetector trace. No ^{14}C-labelled aminobifenox acid or aminobifenox was found in the water sample.

Mineralisation of the reference test item [$^{14}C(U)$]Benzoic acid confirmed sufficient microbial activity of the test water. After 14 days, 83.9% was mineralised in the test system.

The DT_{50} values for [^{14}C]-Bifenox were determined to be 4.5 days (low test concentration) and 3.7 days (high test concentration) in the water phases. The DT_{90} values were determined to be 17.9 days (low test concentration) and 14.4 days (high test concentration) in the water phases.

The mean recovery of radioactivity in the sterile samples was 92.8% AR after 21 days of incubation. One major metabolite (M1 = bifenox acid) was detected accounting for 48.0% AR.

4.1.3.2 Study 2 – CA 7.2.2.3 Water/sediment studies

Study reference:

Report:	Anonymous (1992)
Title:	Degradation and metabolism of Bifenox in water/sediment systems
Document No:	200094, 272700
Guidelines:	BBA IV, 5-1
GLP	yes

Study summary and results:

In the study of Anonymous (1992), investigation of the fate of ^{14}C dichlorophenyl Bifenox in two different water/sediment systems was conducted according to BBA - Guideline IV, 5-1 (December 1990). It must be noted that at the time of evaluation and DAR preparation (November 2003 to July 2005) for inclusion onto Annex I to Directive 91/414 EC, the recommended guideline OECD 307 (adopted 24 April 2002) was 'in force' and had been applied to the evaluation of the study). The water sediment systems comprised a silty loam sand and a silty loam in which organic carbon ranged from 1.3 to 3.5% and pH from 7.5 to 7.8 received an application of 0.33 mg Bifenox/L and were incubated under dark aerobic conditions at 20°C for up to 105 days. Each water/sediment test system contained 2.5 cm sediment overlaying by 6 cm water. Duplicate samples were taken after 0.25, 1, 2, 7, 14, 29, 59 and 105 days after application. Measurements of sediment microbial biomass were performed and increased during the incubation period for one system and decreased in the other. Dissolved oxygen concentrations were above 20% during the study indicating aerobic conditions throughout. Positive redox potential measurements above + 87 mV indicated an oxidising environment in the aqueous phase. Sediment redox potentials were below - 80 mV. Radioactivity in trapping solutions for volatiles was determined and this radioactivity as well as radioactivity from aqueous and sediment samples were quantified by LSC and analysed and identified by TLC and HPLC methods.

The overall recovery of applied radioactivity in both systems ranged from 88.0 to 96.2% AR. Mass balance was slightly below 90% in two samples from one system at 88.8% AR (on day 59) and 88.0% AR (on day 105) and in one sample from the other system at 89.3% AR on day 105. These are not considered to have any relevant effect on the outcome of

the metabolism analysis. Bifenox rapidly disappeared from the two water/sediment systems within the first day. In the water phase, Bifenox appeared at very low levels between 3.5 and 0.2% AR of the applied amount within the first 7 days. Aminobifenox, the major metabolite was bound to the sediment in amounts up to 67% AR of the applied parent compound. No other metabolite was detected in the sediment throughout the study. Aminobifenox occurred to 6.4% AR in the water phase. Aminobifenox acid appeared in one system at a maximum of 12.7% AR in water 24 hours after treatment. This level decreased to 5.2% within the following 24 hours and did not reach amounts above 10% thereafter. In the other system, a maximum amount of 10.6% AR was found on day 14 and decreased to 3.1% AR at the consecutive sampling event. However, at day 105 still 5% AR found in the water accounted for this metabolite. Bifenox acid accounted for maximum 7.8% AR in water 48 hours after application and did not exceed 5% at any other sampling time.

Non-extractable radioactivity in the sediments increased steadily throughout the study and accounted for approximately 63% AR 105 days after application of Bifenox. Less than 5% AR was found to be associated to volatile compounds.

The dichlorophenyl ring radiolabel used in this study is regarded as sufficient to fully describe the degradation of Bifenox in dark water/sediment system. Additional investigation with the nitro label is not necessary since, as demonstrated in hydrolysis study data, bifenox acid was the only metabolite detected at pH 7 and 9. Furthermore, no difference in metabolism of Bifenox was observed when studied with both chloro and nitro labels in aerobic soil route and rate of degradation studies.

Kinetic re-evaluation of the DT values was performed and the half-lives of Bifenox in the total system were 0.02 and 0.06 days in the 'Bickenbach' and 'Unter Widdersheim' system, respectively. For the metabolite bifenox acid, maximum DT_{50} value for the water compartment was 2.54 days (Bickenbach system), and since there was no occurrence in the sediment phase, this endpoint can be extrapolated to the total system as well. For the metabolite aminobifenox acid, for the whole system maximum DT_{50} value was 30.75 days (Bickenbach system). For the metabolite Aminobifenox acid, no appropriate kinetic fitting could be found.

4.1.4 Other degradability studies

4.1.4.1 Study 1 – CA 7.1.1.1 Aerobic degradation

Study reference:

Report:	Anonymous (1999)
Title:	[14C]-bifenox: aerobic route of degradation
Document No:	15745, 202231
Guidelines:	SETAC (1995) Procedure for assessing the environmental fate and ecotoxicity of pesticides
GLP	yes

Study summary and results:

In the study of Anonymous (1999), [chloro phenyl-^{14}C] Bifenox and [nitro phenyl-^{14}C] Bifenox was added to a loam soil (pH 5.7 ($CaCl_2$), OC 2.3%) at a rate of 1.16 mg/kg dry soil, equivalent to 1.16 kg/ha. The soil test systems were incubated under dark aerobic conditions at 20 ± 2°C and a soil water content of 45% maximum water holding capacity. Duplicate replicate samples were taken at each sampling point over a period of up to 120 days of incubation.

All soil samples were extracted once with methanol and once with methanol/water (1:1 v/v). The day 0 to 9 samples underwent an additional extraction with methanol/water Sonic and day 14 samples and onwards underwent an additional Soxhlet extraction. In all cases, the radioactive content was determined by LSC. Volatile degradation products were trapped and quantified and identified using a specific precipitate principle. All extracts were analysed by HPLC with confirmation analysis of selected samples via TLC and LC-MS for identity confirmation. For selected samples, the distribution of radioactivity between humic, fulvic and humic acid fractions was investigated.

The test soil was found to be biologically active with measurements of soil biomass using the fumigation extraction method at test start (238 µg C/g soil) and end (212 µg C/g soil) showing no decrease in microbial viability. The total mean recovery of applied radioactivity (AR) over all sampling dates was 95.0% (chloro phenyl label) and 95.6% (nitro phenyl). Only one sample was outside the 90-110% AR range at day 120 for one replicate for the nitro phenyl label. The recovery

was 89.15% AR and is considered not to have any impact on the study outcome. Recovery in the corresponding duplicate was 94.04% AR leading to a mean recovery of 91.60% AR. Also, at this sampling point for this label, any metabolites observed had already reached peak amounts at earlier sampling times and were in decline.

There were no notable differences in the metabolism and rate of degradation of Bifenox between the two labels. Bifenox was moderately quickly degraded in the soil declining from mean 87.01-90.16% AR at day 0 to 9.04-9.59% AR after 120 days. The mean amount of $^{14}CO_2$ evolved during incubation was initially low and less than 1% AR until day 28 (chloro phenyl label) and day 56 (nitro phenyl label). It increased slowly to reach maximum levels at study end of 11.17% AR and 6.30% AR for chloro phenyl label and nitro phenyl labels, respectively. CO_2 was determined to be the predominate volatile. The level of non-extractable residues increased steadily during incubation reaching maximum values of 43.30% and 46.09% AR (chloro and nitro-phenyl labels, respectively) at study end. They were distributed primarily with humic acid and humin fraction with chloro phenyl labelled samples or humic acid fraction with nitro phenyl labelled samples.

One major metabolite was detected. Bifenox acid was observed accounting for maximum on day 14 at 63.8% AR (chloro phenyl label) and 60.87% AR (nitro phenyl label) and decreased to ca. 27% AR at study end forming bound residues and CO_2. As minor metabolites, aminobifenox and aminobifenox acid occurred to a maximum levels of 1.15% AR (day 2) and 0.84% AR (day 90), respectively with no remarkable difference between the labels.

4.1.4.2 Study 2 – CA 7.1.1.1 Aerobic degradation

Study reference:

Report:	Anonymous (2000)
Title:	[14C]-bifenox rate of aerobic degradation in three soil types at 20°C and in one soil type at 10°C
Document No:	15747, 202232
Guidelines:	SETAC (1995) Procedure for assessing the environmental fate and ecotoxicity of pesticides
GLP	yes

Study summary and results:

Further information on the aerobic soil metabolism of [chloro phenyl-^{14}C] Bifenox is provided in the study of Anonymous (2000). The test substance was added to three different soils – sandy loam (high microbial activity) (pH 5.4 ($CaCl_2$), OC 2.6%), clay loam (pH 7.0 ($CaCl_2$), OC 4.7%), sandy loam (low microbial activity) (pH 5.3 ($CaCl_2$), OC 1.8%) – at a rate of 1.17 mg/kg dry soil, equivalent to 1.17 kg/ha. The soil test systems were incubated under dark aerobic conditions at 20 ± 2°C (and also at 10 ± 2°C for the sandy loam (high microbial activity) soil) and a soil water content of 45% maximum water holding capacity. Single samples were taken at each sampling point over a period of up to 181 days of incubation.

All soil samples were extracted once with methanol and twice with methanol/water (1:1 v/v). Acetonitrile/water Soxhlet extraction was carried out after 1 day for the clay loam soil, after 8 days for the two sandy loam soils incubated at 20°C and after 14 days for the sandy loam soil incubated at 10°C. In all cases, the radioactive content was determined by LSC. Volatile degradation products were trapped and quantified and identified using a specific precipitate principle. All extracts were analysed by HPLC with confirmation analysis of selected samples via TLC and LC-MS for identity confirmation.

The test soil was found to be biologically active with measurements of soil microbial biomass using the fumigation extraction method at test start and end showing no unacceptable decrease in microbial viability. Values in µg C/g soil determined were 413 at test start and 339 (20°C) / 288 (10°C) at test end for sandy loam (high microbial activity); 1134 at test start and 1003 at test end for clay loam; 220 at test start and 229 at test end for sandy loam (low microbial activity).

The total mean recovery of applied radioactivity (AR) over all sampling dates ranged from 94.68% AR (clay loam) to 97.93% AR (sandy loam, high microbial activity, 20°C). The majority of recovered radioactivity was above 90% AR. However, for the soils incubated at 20°C, recovery was below 90% AR on some sampling occasions as follows:

- Sandy loam (high microbial activity), 88.24% AR on day 181.
- Clay loam, 89.85% AR (day 92), 84.44% AR (day 120), 89.27% AR (day 181).
- Sandy loam (low microbial activity), 86.39% AR on day 30

The losses in recovery were thought to be due to inefficiencies of combustion with an elevated amount of Unextractable residue present (> 30% AR) leading to exaggerated errors.

For the sandy loam (high microbial activity), this single recovery slightly below 90% AR on the last sampling day (181) is considered to have no impact on the outcome of the route or rate part of the study. At this time point, over 90% degradation of the parent active had already occurred (by day 92) and major metabolite formation had already peaked at a far earlier time point (day 56) and was steadily decreasing since then.

For the clay loam, the recoveries slightly below 90% AR on the last three sampling days (92, 120 and 181) are considered to have no impact on the outcome of the route or rate part of the study. Already by day 30, over 90% degradation of the parent active had already occurred and major metabolite formation had already peaked at day 10 (78.71% AR) and was subsequently steadily decreasing over the remaining incubation period. On the last three sampling dates, major metabolite amounts were 48.87%, 35.44% and 23.11% AR. It is obvious that the small losses in recovery observed on these days would have no major impact on the amount of major metabolite recovered.

For the sandy loam (low microbial activity), this single recovery slightly below 90% AR on sampling day 30 is considered to have no impact on the outcome of the route or rate part of the study. At this time point, over 50% degradation of the parent active had already occurred (by day 10). Major metabolite formation was seen at 56.26% AR on day 30 and at a maximum of 58.54% AR on the next sampling day 56. The losses in recovery with the small amount of 3.6% AR below the recommended lower part of the recommended 90-110% range is not expected to have any relevance of significance for the reliability of the data generated for this soil. Already in the clay loam, the highest occurrence of the major metabolite was observed at 78.71% AR. The fact that no other metabolites occur to 5% AR or even near this amount in any of the soils evaluated in this study or in the first study by Anonymous (1999) further supports the reliability of the data and the insignificance of this single sample point 3.6% AR below the recommended lower part of the 90-110% range.

Bifenox was moderately quickly degraded in the soils at 20°C declining from 98.07-95.01% AR at day 0 to 2.00-3.78% AR after 181 days. At 10°C, Bifenox degraded from 95.86% AR at day 0 to 10.80% AR at day 181. The amount of $^{14}CO_2$ evolved during incubation was initially low and less than 1% AR until day 30 in all soils at 20°C and until day 92 at 10°C. At 20°C, $^{14}CO_2$ increased slowly to reach maximum levels at study end of 9.61-18.87% AR. At 10°C, $^{14}CO_2$ reached only maximum 1.60% AR at day 181. CO_2 was determined to be the predominate volatile. The level of non-extractable residues increased steadily during incubation reaching maximum values of 34.95-51.72% AR in the soils at 20°C and 19.43% AR in the soil at 10°C.

One major metabolite was detected. Bifenox acid was observed accounting for maximum on day 10 at 78.71% AR (clay loam) and decreased to 23.11% AR at study end forming bound residues and CO_2. As minor metabolites, aminobifenox and aminobifenox acid occurred to a maximum levels of 0.59% AR (day 181) and 2.58% AR (day 120), respectively with no remarkable difference between the soils. Incubation at 10°C resulted in a slower degradation of bifenox and subsequently lower metabolite amounts formed.

4.1.4.3 Study 3 – CA 7.1.1.1 Aerobic degradation

Study reference:

Report:	KCA 7.1.1.1/01 - Anonymous (2013)
Title:	Aerobic and anaerobic transformation of Bifenox in soil with the focus on possible nitrofen formation (includes 1st amendment appended to page 56-57)
Document No:	None
Guidelines:	Study carried out on the basis of OECD 307 (2002)
GLP	no

Study summary and results:

The degradation of non-labelled Bifenox applied at a rate corresponding to 0.720 kg/ha under aerobic conditions was investigated in two different soils, LUFA 2.2 loamy sand and RefeSol 03-G silt loam. Both soils were incubated at standardised aerobic conditions at 20°C in the dark. After appropriate time intervals, soil samples were extracted and analysed for the test and reference compounds. The water content of the soil samples during the incubation was set

between 40 – 45% MWHC. The application rate was calculated to be 0.96 mg a.s. per kg dry soil corresponding to 48 µg a.s. per 50 g soil dry weight.

Replicate soil samples were taken for analyses at days 0, 3, 7, 14, 28, 50, 76 and 90 after application. After sampling, soil and aqueous supernatants, if present, were extracted and analysed for the test and reference substances by High Performance Liquid Chromatography with UV-detection (HPLC-UV) and Liquid Chromatography with high resolution mass detection (LC-MS).

The amounts of Bifenox decreased continuously to concentrations in the range of 12.5% to 13.8% of the initial applied test substance at day 50 after treatment. Afterwards, the Bifenox concentration decreased further (in case of Refesol 03-G) or remained relatively stable until the end of incubation at 90 days (LUFA 2.2). Bifenox acid in soil samples increased continuously until 14 days after application to concentrations in the range of 43.7% to 55.9% demonstrating the transformation of the test substance during aerobic degradation. Afterwards, this main aerobic metabolite remained relatively stable up to 50 days of aerobic incubation. Bifenox acid concentrations decreased thereafter to amounts of 25.8% and 27.7% until the end of incubation. Nitrofen was not detected in concentrations above the LOD of 0.003 mg/kg by means of LC-MS.

4.1.4.4 Study 4 – CA 7.1.2.1.2 Aerobic degradation of metabolites, breakdown and reaction products

Study reference:

Report:	Anonymous (2003)
Title:	Degradation of bifenox acid in three different soils under aerobic conditions at 20 °C in the dark
Document No:	20031161/01-CABJ, 20031161/01-CABJ
Guidelines:	OECD 307
GLP	yes

Study summary and results:

The rate of degradation of bifenox acid was investigated in the study of *Anonymous (2003*) conducted in accordance with SETAC 195 and OECD 307 (2002). The test substance non-radio-labelled bifenox acid was added to three different soils – 2.2 loamy sand (pH 5.7 ($CaCl_2$), OC 2.06%), 2.3 sandy loam (pH 6.1 ($CaCl_2$), OC 1.16%), 3A loam (pH 7.2 ($CaCl_2$), OC 2.87%) – at a rate of 0.64 mg/kg dry soil. The soil test systems were incubated under dark aerobic conditions at 20 ± 2°C and a soil water content of 45% maximum water holding capacity. Duplicate replicate samples were taken at each sampling point over a period of up to 120 days of incubation. Additionally, two similarly incubated control samples, one of them fortified, were also taken. All soil samples were extracted once with acetonitrile/water (80:20 v/v) and analysis with HPLC and MS/MS detection. Method validation for recovery of bifenox acid ranged from 82-101% for all soils. Recovery of samples fortified at the time of sampling of treated flasks ranged from 78.4-130% for all soils.

The test soil was found to be biologically active with measurements of soil microbial biomass based on the short-term respiration method at test start and end showing no unacceptable decrease in microbial viability nor any differences in biomass between treated and untreated soils. Values in mg C/100 g soil determined for treated soils were 32.8 at test start and 18.9 at test end for loamy sand; 8.7 at test start and 6.5 at test end for sandy loam; 113 at test start and 49.0 at test end for loam. Similar ranges were observed in untreated soils incubated under the same conditions.

Bifenox acid was moderately to quickly degraded in the soils at 20°C declining from mean of 98.5-107.7% applied at day 0 to 2.84-35.8% applied after 120 days. The results were corrected for recovery of fortified samples at each sampling time.

4.1.4.5 Study 5 – CA 7.1.2.1.2 Aerobic degradation of metabolites, breakdown and reaction products

Study reference:

Report:	Anonymous (2005a)
Title:	Degradation of aminobifenox in 3 different soils under aerobic conditions at 20 °C in the dark
Document No:	20051048/01-CABJ, 20051048/01-CABJ
Guidelines:	OECD 307 and SETAC (1995)
GLP	yes

Study summary and results:

The rate of degradation of aminobifenox was investigated in the study of *Anonymous (2005a*) conducted in accordance with OECD 307 (2002). The test substance non-radio-labelled aminobifenox was added to three different soils – 2.2 loamy sand (pH 5.6 ($CaCl_2$), OC 2.71%), 3A sandy loam (pH 6.5 ($CaCl_2$), OC 2.42%), 5M sandy loam (pH 6.9 ($CaCl_2$), OC 1.17%) – at a rate of 0.25 mg/kg dry soil. The soil test systems were incubated under dark aerobic conditions at 20 ± 2°C and a soil water content of 45% maximum water holding capacity. Duplicate replicate samples were taken at each sampling point over a period of up to 120 days of incubation. Additionally, two similarly incubated control samples, one of them fortified, were also taken. After shaking soils samples with 100 mL acetonitrile, x mL water (x = 100 mL – water content of soil) and 0.5 g ascorbic acid overnight and sedimentation of soil, about 1-2 mL of the clear supernatant was filtered and transferred to glass vials for analysis. All samples were diluted at least 1:5 with methanol/water/acetic acid (50:50:0.5, v/v/v) before analysis using a HPLC-MS/MS method. Method validation for recovery ranged from 88-101% for all soils. Recovery of samples fortified at the time of sampling of treated flasks ranged from 77-101% for all soils.

The test soil was found to be biologically active with measurements of soil microbial biomass based on the short-term respiration method at test start and end showing no unacceptable decrease in microbial viability nor any differences in biomass between treated and untreated soils. Values in mg C/100 g soil determined for treated soils were 42.6 at test start and 20.7 at test end for 2.2 loamy sand; 77.8 at test start and 53.1 at test end for 3A sandy loam; 27.8 at test start and 19.3 at test end for 5M sandy loam. Similar ranges were observed in untreated soils incubated under the same conditions.

Aminobifenox was moderately to rapidly degraded in the soils at 20°C declining from mean of 101.8-105.1% applied at day 0 to 3.2-4.0% applied at end of incubation. The results were corrected for recovery of fortified samples at each sampling time.

4.1.4.6 Study 6 – CA 7.1.2.1.2 Aerobic degradation of metabolites, breakdown and reaction products

Study reference:

Report:	Anonymous (2005b)
Title:	Degradation of aminobifenox acid in 3 different soils under aerobic conditions at 20 °C in the dark
Document No:	20051049/01-CABJ, 20051049/01-CABJ
Guidelines:	OECD 307 and SETAC (1995)
GLP	yes

Study summary and results:

The rate of degradation of aminobifenox acid was investigated in the study of *Anonymous (2005b*) conducted in accordance with OECD 307 (2002). The test substance non-radio-labelled AMINOBIFENOX acid was added to three different soils – 2.2 loamy sand (pH 5.6 ($CaCl_2$), OC 2.71%), 3A sandy loam (pH 6.5 ($CaCl_2$), OC 2.42%), 5M sandy loam (pH 6.9 ($CaCl_2$), OC 1.17%) – at a rate of 0.25 mg/kg dry soil. The soil test systems were incubated under dark aerobic conditions at 20 ± 2°C and a soil water content of 45% maximum water holding capacity. Duplicate replicate samples were taken at each sampling point over a period of up to 62 days of incubation. Additionally, two similarly incubated control samples, one of them fortified, were also taken. After shaking soils samples with 100 mL acetonitrile, x mL water (x = 100 mL – water content of soil) and 1 mL ammonia (28%) overnight and sedimentation of soil, about 1-2 mL of the clear supernatant was filtered and transferred to glass vials for analysis. All samples were diluted at least 1:5 with methanol/water/acetic acid (50:50:0.5, v/v/v) before analysis using a HPLC-MS/MS method. Method validation for recovery ranged from 87-95% for all soils. Recovery of samples fortified at the time of sampling of treated flasks ranged from 76-102% for all soils.

The test soil was found to be biologically active with measurements of soil microbial biomass based on the short-term respiration method at test start and end showing no unacceptable decrease in microbial viability nor any differences in biomass between treated and untreated soils. Values in mg C/100 g soil determined for treated soils were 43.1 at test start and 19.9 at test end for 2.2 loamy sand; 80.4 at test start and 52.1 at test end for 3A sandy loam; 27.1 at test start and 19.6 at test end for 5M sandy loam. Similar ranges were observed in untreated soils incubated under the same conditions.

Aminobifenox acid was rapidly degraded in the soils at 20°C declining from mean of 85.1-103.9% applied at day 0 to 1.9-4.5% applied at end of incubation. The results were corrected for recovery of fortified samples at each sampling time.

Evaluation of DT values

The rate of degradation of Bifenox has been evaluated in two aerobic soil laboratory studies. The relevant DT_{50} values from the available studies were re-evaluated according to recommendations of the FOCUS workgroup on degradation kinetics (2006)[1] and also normalised to 20°C and pF2, where required, in order to derive new DT_{50} values for use as persistence and modelling endpoints. Persistence DT_{50} endpoints of Bifenox at 20°C (not normalised to pF2) ranged from 3.96 to 14.64 days (n=4) and respective DT_{90} values ranged from 13.17 to 116.90 days. Modelling DT_{50} endpoints at 20°C and pF2 ranged from 2.49 to 16.48 days (geometric mean of 7.56 days, n=4).

The same studies for the parent also provided rate data for the metabolite bifenox acid. And in separate studies, the rate of degradation of non-radio-labelled bifenox acid as well as aminobifenox acid (and the minor metabolite aminobifenox) was also investigated. An evaluation of all data according to FOCUS kinetics was made.

Resulting persistence DT_{50} endpoints of bifenox acid at 20°C (not normalised to pF2) ranged from 22.65 to 165.27 days (n=7) and respective DT_{90} values ranged from 75.26 to 549.01 days. Modelling DT_{50} endpoints at 20°C and pF2 ranged from 17.64 to 120.81 days (geometric mean of 42.83 days, n=7). The highest value always occurred in a soil with low microbial content. The same soil with a high microbial content had persistence DT_{50} and DT_{90} values of 60 and 199 days and a modelling DT_{50} of 43.86 days.

For aminobifenox acid, persistence DT_{50} endpoints at 20°C (not normalised to pF2) ranged from 0.55 to 1.58 days (n=3) and respective DT_{90} values ranged from 10.94 to 28.81 days. Modelling DT_{50} endpoints at 20°C and pF2 ranged from 1.79 to 7.24 days (geometric mean of 4.01 days, n=3).

For aminobifenox, persistence DT_{50} endpoints at 20°C (not normalised to pF2) ranged from 4.89 to 6.37 days (n=3) and respective DT_{90} values ranged from 16.24 to 37.70. Modelling DT_{50} endpoints at 20°C and pF2 ranged from 3.57 to 9.47 days (geometric mean of 5.40 days, n=3).

[1] FOCUS (2006) "Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration" Report of the FOCUS Work Group on Degradation Kinetics, EC Document Reference Sanco/10058/2005 version 2.0, 434 pp

4.1.4.7 Study 7 – CA 7.1.1.2 Anaerobic degradation

Study reference:

Report:	KCA 7.1.1.2.1/01 - Anonymous (2016a)
Title:	Anaerobic transformation of Bifenox in soil
Document No:	ADM-001/5-31, 90017808
Guidelines:	OECD 307 (2002)
GLP	yes

Study summary and results:

Degradation of Bifenox was investigated under anaerobic conditions at 20°C in the dark in one soil. The study was performed with ^{14}C-[dichlorophenyl]Bifenox applied onto the soil at a rate corresponding to 0.72 kg/ha. After an aerobic incubation phase of 6 days, samples were incubated under anaerobic conditions at 20 ± 2°C in the dark to up to 120 days after application (DAA).

Bifenox was added to each glass vessel (containing 50 g soil). During the aerobic phase soil moisture content was adjusted to 50% MWHC and gas exchange was realised by means of a flow-through incubation system. Anaerobic incubation conditions were established by flooding the soil samples with purified water after an aerobic incubation phase and change of the flow-through gas from synthetic air to nitrogen. The outflow from the incubation vessels was passed through different solutions in order to trap possible volatile degradation products.

Microbiological activity of the test soil was monitored at start, during and at the end of the study and results indicated satisfying biological activity over the incubation period. Replicate soil samples were taken for analyses at 0, 3, 6, 14, 21, 30, 62, 90 and 120 DAA. Soil samples were extracted and extracts and aqueous supernatants were analysed for the test substance and possible degradation products by radio-HPLC and LC-MS/MS.

Mass balance was established for all samples. The values ranged from 98.4% - 91.5% AR (applied radioactivity) for the samples up to 62 days of incubation. However, for the last two sampling points the mass balance for three samples was slightly below 90% AR, leading to mean mass balance of 89.1% AR (90 d) and 87.3% AR (120 d) and giving an overall range of 98.4% - 87.3% AR for the complete sample set.

The total extractability of radioactive residues at start of the incubation was 98.1% AR. The extractability decreased thereafter with time down to 32.4% AR at the end of the incubation phase. Bound radioactive residues increased throughout the incubation, reaching a maximum value of 54.4% AR at the end of incubation (120 d). Only negligible amounts (< 1% AR) of volatile degradation products were found in the trapping solutions.

Besides parent Bifenox, three metabolites were detected by radio-HPLC analyses. The amounts of Bifenox decreased quickly to 33.9% AR at day 6 after treatment (start of the anaerobic phase). Afterwards, the Bifenox amount decreased further. No Bifenox was detected after 90 d of incubation.

Bifenox acid built up during the aerobic incubation phase and increased to a concentration of 49.3% until 6 days after application of Bifenox. Afterwards, under anaerobic conditions, this major aerobic metabolite was further degraded to aminobifenox acid. No bifenox acid was detected any more from day 30 onwards. Beginning from day 14 after application, aminobifenox acid was detected indicating that amination takes place under anaerobic conditions. The amounts of aminobifenox acid increased slowly reaching maximum of 29.3% AR after 90 days of incubation and thereafter decreasing to 24.0% AR at the final sampling at day 120. Aminobifenox was detected in some samples but without a clear temporal trend and not at consecutive samplings with a maximal value of 8.6% AR observed after 30 days. Nitrofen was never detected (LOQ of the method 0.01 mg/kg; LOD: 0.003 mg/kg).

Half-lives of parent Bifenox and the metabolites were calculated by KinGUI (version 1.1) assuming hockey stick (HS) kinetic model for parent Bifenox and single first order (SFO) kinetic model for the metabolites. The calculated DT_{50} values considering simultaneous fitting were 9.9, 12.8 and 224.4 days for Bifenox, bifenox acid and aminobifenox acid, respectively and the respective DT_{90} values were 68.0, 42.6 and 745.4 days.

4.1.4.8 Study 8 – CA 7.1.1.2 Anaerobic degradation

Study reference:

Report:	KCA 7.1.1.2/02 - Anonymous (2013)
Title:	Aerobic and anaerobic transformation of Bifenox in soil with the focus on possible nitrofen formation (includes 1st amendment appended to page 56-57)
Document No:	None
Guidelines:	Study carried out on the basis of OECD 307 (2002)
GLP	No

Study summary and results:

The degradation of non-labelled Bifenox under anaerobic conditions was investigated in 1 soil, LUFA 2.2 loamy sand, at a rate corresponding to 0.720 kg a.s./ha. The soil was incubated at 20°C in the dark, initially under aerobic conditions for 7 days (corresponding to approximately one half-life of Bifenox) followed by standardised anaerobic conditions in a flow-through system. Before start of the incubation, the moisture content was adjusted to about 40-50% of its maximum water holding capacity (WHC_{max}).

Samples of the soil were prepared by placing 50 g soil, based on dry weight, in glass centrifuge tubes. Application of Bifenox was performed using an acetonitrile solution. The solution was pipetted onto the soil samples which then were mixed carefully. At 7 days after application the soil samples were flooded with purified water and the incubation system was flushed with nitrogen to establish and maintain anaerobic conditions. Replicate soil samples were taken for analyses at days 0, 7, 10, 11, 14, 28, 49, 73 and 90 after application. After sampling, soil and aqueous supernatants, if present, were extracted and analysed for the test and reference substances by High Performance Liquid Chromatography with UV-detection (HPLC-UV) and Liquid Chromatography with high resolution mass detection (LC-MS).

Bifenox determined by HPLC-UV analyses decreased continuously to concentrations in the range of 7.6% to 8.8% of the initial applied test substance at day 49 after treatment. Bifenox acid increased continuously until 11 days after application (day 2 of anaerobic conditions) to concentrations in the range of 47.0% to 48.9% demonstrating the transformation of the test substance under the initial aerobic conditions. It was not detected anymore from the next sampling day (day 14) onwards. Small amounts of amino-bifenox acid (maximum 8.9% of initially applied Bifenox) were detectable from day 14 until day 49 of incubation indicating that amination takes place under anaerobic conditions. Nitrofen was not determined by LC-MS analysis in concentrations above the LOQ of 0.008 mg/kg. Other possible metabolites (e.g. amino-bifenox) were not looked for as this was not within the scope of this study.

4.1.4.9 Study 9 – CA 7.1.1.3 Soil photolysis

Study reference:

Report:	Anonymous (1999)
Title:	[14C]-Bifenox: Photodegradation on soil
Document No:	15750, 202111
Guidelines:	EPA 161-3
GLP	yes

Study summary and results:

The soil photolysis of Bifenox has been evaluated in one study (Anonymous, 1999) which has been summarised in the DAR of Bifenox prepared for the first EU evaluation for Annex I inclusion of Bifenox. In the study conducted according to US EPA, Subdivision N, Paragraph 161-3 (1982), [chloro phenyl-^{14}C] Bifenox was added to a loam soil (pH 5.7

($CaCl_2$), OC 2.3%) at a rate equivalent to 1.26 kg CA/ha. The soil test systems were incubated under aerobic conditions at 20 ± 1°C and a soil water content of 45% maximum water holding capacity for up to 30 days. The soil received irradiation for 13.27 hours per day from a xenon light source with UV filter. Non-irradiated soil samples were run concurrently. Duplicate samples were taken at each sampling point over a period of up to 30 days of incubation.

All soil samples were extracted once with methanol/water (50:50 v/v) followed by methanol and followed by Soxhlet extraction for some samples from day 7 onwards. In all cases, the radioactive content was determined by LSC and extracts were analysed by HPLC. Selected samples were characterised by TLC. Volatile degradation products were trapped, quantified and identified. Aliquots of soil samples containing less than 10% radioactivity were reduced in volume prior to determining radioactivity by LSC. Unextracted residues were combusted prior to quantification.

Mass balance was seen to be between 93 and 104.3% AR for both irradiated and non-irradiated soils. After 30 days, non-extracted residues were at maximum of 10.8 and 16.2% AR for irradiated and non-irradiated soils, respectively. The metabolism observed was similar between irradiated and non-irradiated soils with, respectively, 57.5 and 40.1% AR remaining after 30 days as parent Bifenox and 16.5 and 28.2% AR recovered as bifenox acid. According to first order kinetics, DT_{50} / DT_{90} values determined for irradiated and non-irradiated soils were, respectively, 41.3 / 137.2 days and 23.8 / 79.2 days. In conclusion, photolysis was found not to have any significant contribution to the degradation of Bifenox on soil surfaces.

4.1.4.10 Study 10 – CA 7.1.2.2.1 Field soil dissipation studies

Study reference:

Report:	Anonymous (1982)
Title:	Bifenox - Terrestrial field dissipation
Document No:	440163, 440163
Guidelines:	not stated
GLP	no

Study summary and results:

The field soil dissipation of Bifenox has been evaluated in one study (**Anonymous, 1982**) which has been summarised in the DAR of Bifenox prepared for the first EU evaluation for Annex I inclusion of Bifenox. The study covered application to bare soil in four sites in the USA (Florida, Nebraska, Virginia and New Jersey) where applications were made between April and July. Using the residue levels of parent Bifenox determined over the whole core sampled (either 0-15 or 0-8 cm (New Jersey) soil layer), single first order DT_{50} of 8.3-32.1 days were determined. The study however provided no information on potential metabolites formed and lacked key information on soil properties and analytical procedure.

4.1.4.11 Study 11 – CA 7.1.2.2.1 Field soil dissipation studies

Study reference:

Report:	KCA 7.1.2.2.1/01 - Anonymous (2016)
Title:	Field soil dissipation study with one autumn application of FOX (AG-B2-480 SC) a formulated product containing Bifenox on 3 sites in north Europe and 2 sites in south Europe in 2014-2015
Document No:	S14-04459, R-90017806
Guidelines:	SETAC 1995 SANCO/3029/99 rev. 4: EU guidance document for generating and reporting methods of analysis in support of pre-registration data requirements. EPA (Oct 2008). Fate, Transport and Transformation Test Guidelines. OPPTS 835.6100. Terrestrial Field Dissipation EFSA Guidance Document for evaluating laboratory and field dissipation studies to obtain DegT50 values of active substances of plant protection products and transformation products of these active substances in soil. EFSA Journal 2014;12(5): 3662. OECD 217
GLP	yes

Study summary and results:

In the study, the residue levels of Bifenox and its metabolite bifenox acid were determined in five trials at different sites in northern Europe (Trial 1 N-Germany, Trial 5 S-Germany and Trial 2 N-France) and southern Europe (Trial 3 S-France and Trial 4 Spain). In addition, analysis for nitrofen was carried out to investigate if it can be formed under the conditions reflecting good agricultural practice for the use of Bifenox-based products. One treated plot divided into three equal sized subplots and one control plot were installed at each trial site. A single autumn application of FOX (480 g/L Bifenox) was applied to bare soil at nominal application rate of 757.5 g a.s./ha. Immediately after application, the soil surface was covered with a sand layer of 0.5 cm thickness. Soil samples were collected at day 0 (0-30 cm) and 11 additional timings (0-100 cm). After collection soil cores were bagged, labelled and deep frozen. Soil characterisation and soil bulk density samples (0-100 cm), water holding capacity samples as well as residue samples (0-100 cm) were taken 0-12 days before application (DBA). The soil microbial biomass sample (0-20 cm) was taken 0-11 DBA. For each sampling interval, soil cores were cut into 10 cm layers (0-10 cm, 10-20 cm, 20-30 cm and further if needed) in deep frozen state and homogenised by milling with dry ice.

Specimen extraction and determination of residues was performed according to the multi-residue method and quantification was performed by use of LC-MS/MS detection for bifenox and bifenox acid and by use of GC-MS and LC-MS/MS for nitrofen. The limit of quantification (LOQ) of the analytical method was 0.01 mg/kg for each analyte in soil with a limit of detection (LOD) set at 0.003 mg/kg (30 % of the LOQ).

In treated soil samples of all trials, residues of Bifenox were determined between 0.21 mg/kg and 0.52 mg/kg (as wet weight) 0 days after application (DAA) in the 0 to 10 cm layer. In all trials, residue amounts decreased to negligible levels by study end and were rarely found in relevant amounts in lower soil layers throughout the study. A maximum of 0.063 mg/kg was observed in the 10 – 20 cm soil layer in Trial 3 and a maximum of 0.027 mg/kg in the 20 – 30 cm layer at 7 DAA. These findings were likely due to preferential flow following a dry period leading to cracking of the loamy-textured soil.

Residues of bifenox acid ranged from < LOD to 0.018 mg/kg at 0 DAA. The residues of bifenox acid increased to a maximum between 0.14 mg/kg (Trial 1 at 90 +/- 3 DAA) to 0.23 mg/kg mg/kg (Trial 5 at 60 +/- 3 DAA) in the 0 to 10 cm layer. In the 10 to 20 cm layer a maximum from 0.035 mg/kg (Trial 2 at 120 +/- 3 DAA) to 0.095 mg/kg (Trial 4 at 60 +/- 3 DAA) was observed. Bifenox acid was not detected in any samples from soil layers below 30 or 40 cm.

In all trials, no residues of nitrofen were detected (LOD < 0.03 mg/kg).

A kinetic evaluation of the five available trial datasets was performed to derive trigger and modelling endpoints of Bifenox and its metabolite bifenox acid according to the guidance of the FOCUS work group on degradation kinetics and EFSA. The derived DT_{50} / $DegT_{50}$ values (trigger and modelling endpoints – modelling endpoints normalised to 20°C and pF2) for Bifenox ranged from 8.8 (SFO) to 51 days (DFOP) and from 11.2 (SFO) to 23.3 days (SFO), respectively. For the

metabolite bifenox acid, the derived DT_{50} / $DegT_{50}$ values (trigger and modelling endpoints – modelling endpoints normalised to 20°C and pF2) were ranging from 60.8 to 110 days (both SFO) and from 24.4 to 43.4 days (both SFO), respectively.

4.1.4.12 Study 12 – CA 7.1.2.2.1 Field soil dissipation studies

Study reference:

Report:	KCA 7.1.2.2.1/02 - Anonymous (2015)
Title:	Determination of the storage stability of Bifenox, bifenox-acid and nitrofen in soil under deep frozen conditions
Document No:	S14-04460, 90017807
Guidelines:	EC guideline 7032/VI/95 rev. 5 OECD 506, 2007
GLP	yes

Study summary and results:

The storage stability of bifenox, bifenox acid and nitrofen was investigated by fortifying deep-frozen soil samples with the test items and analysing the samples after a period of storage (12 months) in the deep-freezer. After 12 months of storage, > 70% of the initial concentration of the test items were found in spiked soil samples. Recovered mean concentrations ranged from 85 to 101% applied amount. According to EC guideline 7032/VI/95, OECD 506 from 16 October 2007 and U.S. EPA guideline OPPTS 860.1380, residues can be regarded as stable if the mean recovery over a given storage period does not fall below 70% of the initial value. The results indicate that the test items are stable when stored deep-frozen at < -18°C in soil for at least 12 months of storage.

In addition, one blank control sample for each test item and two freshly fortified recovery samples at 0.01 mg/kg of each test item (separate systems) were analysed at the analysis date. Recovered mean concentrations ranged from 90 to 93% applied amount.

4.1.4.13 Study 13 – CA 7.1.2.2.1 Field soil dissipation studies

Study reference:

Report:	KCA 7.1.2.2.1/03 - Anonymous (2016)
Title:	Degradation and transformation of [dichlorophenyl ring-U-^{14}C] Bifenox in two soils with and without crop (wheat) under greenhouse conditions
Document No:	AS422, 90018635
Guidelines:	OECD 307 (2002)
GLP	yes

Study summary and results:

The route and rate of degradation of [dichlorophenyl ring-U-^{14}C] Bifenox under greenhouse conditions was determined in two French soils in pots with and without crop. Different conditions were established to study the metabolism of Bifenox and check whether nitrofen can be formed. A single application of ^{14}C-labelled test item was applied to soil (incorporated or applied to the soil surface) with and without crop (wheat) at an application rate of 720 g a.s./ha. Soils were incubated under greenhouse conditions for a period of 91 days. Soil samples were collected at day 0 and 2, 7, 10,

20, 30, 62 and 90 days after application (DAA). One system with test item incorporated in soil was also established for mass balance and a single sample was taken at 7, 30, 63 and 91 DAA.

Residues of Bifenox were extracted with acetonitrile measured by LSC for its radioactivity. Samples were then pooled, concentrated and analysed by HPLC and TLC. Where non-extractables accounted for at least 20%, the samples were extracted by acetonitrile under reflux at 80°C in order to check possible formation of metabolites. The samples from the last interval were additionally reflux extracted under neutral conditions before acidic extraction, then submitted to organic matter fractionation.

The total recoveries in terms of percent of the applied radioactivity (AR) from the closed test systems used for material balance ranged between 95.8% and 104.9% for both soils.

The amount of total extractable radioactivity (sum of ambient and reflux extracts) decreased over time from initial levels of 97.6% - 100.6% AR immediately after treatment to 6.5% - 42.0% after 91 days of incubation. Soil pots with seeds and germinated wheat showed a higher decrease in extractable radioactivity at room temperature when compared to the samples without cereals. Under harsh reflux conditions, approximately similar but small amounts were released from all parts. The total extractable radioactivity of the mass balance samples decreased to 24.1% (soil I) and 48.2% AR (soil II) on day 91 after application.

The non-extractable radioactivity increased continuously to maximum of 70.9% AR on day 91 after application. Soil pots without plants showed lower non-extractable residues. Mineralization of the radioactive residues in the closed systems reached a maximum level of 3.6% on day 91. Other volatile products never exceeded 0.1% during the 91-day incubation period.

The amount of Bifenox applied to soil pots steadily decreased and reached less than 10% after 20-30 days of incubation. Bifenox acid was the major metabolite reaching maximum amounts of 56.2% AR (Village-neuf, part III) and 77.1% AR (Bourg-en-Bresse, part II) ten days after application.

Besides the parent compound and bifenox acid, numerous degradation products were detected. Aminobifenox was detected only in small amounts in the extracts of all parts but never exceeded 4.0% AR. Aminobifenox acid was detected in irregular time intervals and was below 5% AR in soil extracts of soil Village-neuf and for at least in three successive time intervals towards the end of the study in the extracts of all test parts of soil Bourg-en-Bresse. 2,4-Dichlorophenol was detected only in one sample and amounted to only 1.4% AR. Nitrofen was never detected in the soil extracts of any part (LOD < 0.01% AR). Up to five other unknown polar metabolites were detected and were very minor. One of the minor fractions showed very similar chromatographic behaviour as the reference standard nitrofen by HPLC and TLC acidic solvents. However, the chromatograms did not always show a 100% co-chromatography. Based on these results, further additional analytical methods were developed to confirm the presence of nitrofen as radioactive substance. The new methods clearly separated the radioactive fraction from nitrofen analytical standard and showed a different polarity.

An unknown fraction N.I.6 was significant in selected samples (up to 14.7% AR). This fraction seems to be an artefact as lower amounts were extracted than expected from both soils and might be formed due to the high concentration of the samples.

The degradation rate of Bifenox and its metabolite bifenox acid in soil was calculated according to FOCUS kinetics guidance (2006) using the software CAKE v. 3.1. Bifenox was rapidly degraded in soil, with SFO DT_{50} values ranging from 1.2 - 4.7 days with formation of the polar metabolite bifenox acid which is further degraded to form mainly bound residues. Bifenox acid was degraded with SFO DT_{50} values ranging from 12.5 - 80 days. Biphasic kinetics generally showed a statistically and visually better fit with DT_{50} values similar to SFO and slightly longer DT_{90} values. However, it should be pointed out that the slow phase occurred once over 90% of Bifenox was degraded.

4.1.4.14 Study 14 – CA 7.2.1.1 Hydrolytic degradation

Study reference:

Report:	Anonymous (2000a)
Title:	[14C]-Bifenox: Hydrolysis under laboratory conditions at pH 4, 5, 7 and 9
Document No:	RNP 636/002253, 202529
Guidelines:	US EPA Guideline 161-1
GLP	yes

Study summary and results:

The study was conducted according to US EPA Guideline 161-1 (meeting also the essential criteria of OECD 111). The hydrolysis of ^{14}C-chloro-phenyl Bifenox in sterile buffer solutions in the dark was investigated in preliminary tests at pH 4, 5, 7 and 9 at 50°C and in a main test at pH 7 and 9 at 25°C. The nominal concentration of the test item was 0.19 mg/L. Achieved measured concentrations were 0.15 to 0.17 mg/L. Single samples were taken at 0, 2 and 5 days in the preliminary test. In the main test at pH 7, single samples were taken on 11 occasions from day 0 up to day 98. In the main test at pH 9, single samples were taken on 12 occasions from day 0 up to day 15. The radioactivity was detected with LSC and the quantity of Bifenox determined with HPLC analytical methods.

The recovery of radioactivity was 102.9 to 104.1% AR for all preliminary and main tests and pH values. In the preliminary test, there was no hydrolysis of Bifenox at pH 4 and 5 after five days at 50°C. At pH 7, 22.1% AR was present as Bifenox after 5 days. At pH 9, Bifenox was completely hydrolysed after 2 days. Bifenox acid was formed to 22.1% AR and 114.9% AR after 5 days at pH 7 and 2 days at pH 9, respectively. In the main test, at pH 7, Bifenox declined from 100% at day 0 to 77% at day 98. At pH 9, Bifenox declined to 6.7% at 15 days. The corresponding first order hydrolysis rate constant was determined and equivalent to a DT_{50} of 265 days and 4 days at pH 7 and 9, respectively. Bifenox acid was the only metabolite detected and occurred at maximum amounts at end of incubation of 21.6% AR at pH 7 and 102.1% AR at pH 9.

Although only one sample was taken, the results are considered as acceptable taking into account the quantity of sampling dates during the main test at each pH. The study is considered as reliable to continue supporting the data requirement for hydrolytic degradation of parent Bifenox and no additional data is required.

4.1.4.15 Study 15 – CA 7.2.1.1 Hydrolytic degradation

Study reference:

Report:	KCA 7.2.1.1/01 - Anonymous (2016b)
Title:	Hydrolysis of bifenox acid as a function of pH
Document No:	ADM-002/1-35, 90018351
Guidelines:	OECD 111, 2004
GLP	yes

Study summary and results:

The study determined the hydrolysis behaviour of bifenox acid at pH 4, 7 and 9, at 50°C in sterile aqueous solution. Bifenox acid was determined to be hydrolytically stable in all pH conditions after 5 days. Based on the results, the DT_{50} at 25°C was estimated to be > 1 year and therefore no additional testing was required.

4.1.4.16 Study 16 – CA 7.2.1.2 Direct photochemical degradation

Study reference:

Report:	Anonymous (2000b)
Title:	Bifenox: Aqueous photolysis
Document No:	RNP 635/003750, C010536
Guidelines:	SETAC (1995) Procedure for assessing the environmental fate and ecotoxicity of pesticides
GLP	yes

Study summary and results:

The test item was dissolved with < 1% acetonitrile in sterile aqueous solution buffered at pH 5 with resulting test concentration of 0.15 g/L. The test systems were incubated at 20°C under continuous irradiation for 72 hours in a Heraeus suntest apparatus with xenon arc light set to an intensity of 275 W m^{-2}. Results were compared with control samples incubated in the dark under similar conditions. Single samples were taken at up to nine intervals over the 72-hour incubation period.

Total recoveries ranged from 93.9 to 103.4% AR for the irradiated system and from 99.1 to 107.7% AR for the dark controls. Analysis was with LSC and HPLC. Sterility of the system was checked and confirmed. No degradation was observed in the dark controls. Under light, a half-life of 24.4 hours was determined. The main photolysis product was 2,4-dichlorophenol accounting for 79.9% AR at study end. However, an artefact – 2,4-dichlorophenol acetate – was produced under the conditions of the test by acetylation of the 2,4-dichlorophenol with the pH 5 acetate buffer. After 72 hours, 15.9% AR was present as 2,4-dichlorophenol and 63.2% AR present as the artefact (total 79.9% AR). Remaining photolytical degradates comprised low levels (≤ 2.8% AR) of volatile radioactivity and polar material.

Although Bifenox was demonstrated to be readily degradable by direct phototransformation, the study does not provide information on the degradation potential of the nitro label. Furthermore, borosilicate vessels were used instead of quartz, absorbance < 340 nm is interfered and bifenox has a peak of absorbance at 300 nm and single samples rather than duplicates were taken.

4.1.4.17 Study 17 – CA 7.2.1.2 Direct photochemical degradation

Study reference:

Report:	KCA 7.2.1.2/01 - Anonymous (2016c)
Title:	Phototransformation of Bifenox in Water – Direct Photolysis
Document No:	ADM-001/5-40, 90017808
Guidelines:	OECD 316, 2008
GLP	yes

Study summary and results:

The aqueous phototransformation of the test item Bifenox was studied under continuous artificial light for up to 168 hours in sterile aqueous media. In tier 1 "Theoretical Screening" the UV/VIS-spectra (290 nm – 800 nm) of non-labelled Bifenox were recorded in buffer solutions of different pH values (pH 4, 7, and 9). The absorption showed a maximum at 300 - 301 nm and showed no difference between pH values. As a consequence to the screening results, the irradiation experiments were conducted at pH 4, due to the hydrolytical stability of Bifenox at acidic conditions. The shortest theoretical environmental half-life of 3.54×10^{-4} days (0.0085 hours) at pH 4 in June at normal climatic conditions indicated a fast photolytical degradation of the test item and met the requirements for a photolysis experiment.
In the main test (tier 2), the irradiation was carried out with three different test item concentrations (nominal 0.1, 0.7 and 1.7 mg/L) for each of the two different label positions in buffer solution at pH 4. The test was performed under sterile

conditions within two irradiation durations of 48 hours and 168 hours (7 days – this extended time to determine metabolite formation). The total mass balances in all samples (including dark control samples) ranged from 94.9 to 105.8% AR (overall mean 99.9% AR). In the aqueous solutions a good recovery was observed only for the middle and low Bifenox concentrations for both labels. In case of the high test concentration, significant amounts of radioactivity exceeding 10% AR in several samples were found adsorbed to the glass of the reaction vessel. As the photolytical behaviour of Bifenox adsorbed on the glass of the reaction vessel was most probably different compared to Bifenox in aqueous solution, the samples applied with high Bifenox concentration were not considered for further evaluation.
Bifenox decreased over time to 16% AR of [dichlorophenyl-^{14}C]-labelled Bifenox and 4% AR of [benzoyl-^{14}C]-labelled Bifenox at both concentrations after 168 hours irradiation. The dark control samples of Bifenox were stable during the whole experimental duration (168 h).
Two different radio-TLC-methods were used as primary identifications methods and HR-LC-MS/MS was used as a confirmatory method. Two transformation products – one from each label of the test item – were found in the irradiated solutions. 2,4-Dichlorophenol was an expected main degradation product from the dichlorophenyl-^{14}C-label and Methyl-5-hydroxy-2-nitrobenzoate was a new degradation product identified from [benzoyl-14C] labelled Bifenox and its identity was confirmed by HR-LC-MS/MS.
A number of unknown metabolites were observed, none of which exceeded 10% AR. Those unknown minor products did not correspond to any available reference standard, but were not further characterised due to their low levels of applied activity.
The direct photolysis rate constant of Bifenox was determined using a single first order (SFO) kinetic model (KinGUI version 1.1). The $DegT_{50}$ values were 63.4, 80.3, 22.9 and 43.9 hours in [dichlorophenol-^{14}C] label (middle rate), [dichlorophenol-^{14}C] label (low rate), [benzoyl-^{14}C] label (middle rate) and [benzoyl-^{14}C] label (low rate), respectively. Corresponding half-lives after conversion into days of natural summer sunlight in June ranged from 1.34 to 4.66 days and in December from 28.3 to 98.8 days.
The quantum yield was calculated after measuring the spectral photon irradiance of the light source and the molar decadic absorption coefficient and ranged from 7.60×10^{-5} to 2.67×10^{-4}.
For the metabolites, half-lives for middle and low concentrations of 43.0 and 267.1 days were calculated for 2,4-dichlorophenol, respectively. For methyl-5-hydroxy-2-nitrobenzoate, half-lives for middle and low concentrations of 63.0 and 56.6 days were calculated, respectively.

4.2 Bioaccumulation

4.2.1 Bioaccumulation test on fish

4.2.1.1 Study 1 - CA 8.2.2.3 Bioaccumulation test on fish

Study reference

Report	Anonymous (1986)
Title	Uptake, depuration and bioconcentration of 14C-bifenox by bluegill sunfish (*Lepomis macrochirus*)
Document No	440205
Guideline	ASTM, proposed standard practise for conducting bioconcentration with fish, 1977 - 1979
GLP	Yes

Study summary and results:

The study was submitted and evaluated for the Annex I inclusion of Bifenox. It is in compliance with GLP regulations and with the current requirements and therefore still valid and appropriate.

The uptake, bioconcentration and depuration of Bifenox in bluegill sunfish was investigated during a 28 days exposure and 14 days depuration period. Bifenox in acetone was continuously dosed to the water flow to a nominal final concentration of 5.0 µg/L. A control only spiked with acetone was set up accordingly. Water concentrations measured over the whole exposure period of 28 days in average were 4.8 µg/L, ranging between 2.4 and 6.2 µg/L. Bifenox accumulates in bluegill sunfish if exposed to constant concentrations. A rapid elimination of ^{14}C Bifenox related residues

from fish was recognised, the DT_{50} for clearance was 1.4 days. The uptake rate constant (k_1) - 650, clearance rate constant (k_2) - 0.51 and steady-state bioconcentration factor (BCF) 1500 for whole fish were determined using a non-linear kinetic modelling computer program for the bioconcentration study with ^{14}C Bifenox. Time to reach 90 % of steady state: 4.5 days. No substance related mortality or abnormal behaviour was observed in the whole study.

4.2.2 Bioaccumulation test with other organisms

Not relevant.

4.3 Acute toxicity

4.3.1 Short-term toxicity to fish

4.3.1.1 Study 1 - CA 8.2.1 Short-term toxicity to fish

Study reference

Report	Anonymous (1993)
Title	The acute toxicity of Bifenox to rainbow trout (*Oncorhynchus mykiss*)
Document No	282/388, 432068
Guideline	OECD 203 (1984)
GLP	Yes

Study summary and results:

The study was submitted and evaluated during first EU evaluation for Annex I inclusion of Bifenox. It was performed in compliance with Good Laboratory Practice (GLP) regulations and according to the current guideline at the time of study conduction. It is still considered acceptable and the conduction of further vertebrate studies is considered inappropriate for animal welfare reasons.

The acute toxicity of Bifenox in rainbow trout was investigated under dynamic conditions for 96 hours. The LC_{50} (96 h) of Bifenox for rainbow trout was determined to be 0.67 mg/L. No deaths occurred at test concentrations of 0.32 mg/L and below. The NOEC was 0.18 mg/L.

4.3.1.2 Study 2 - CA 8.2.1 Short-term toxicity to fish

Study reference

Report	Anonymous (1985a)
Title	Acute toxicity of Bifenox to bluegill (*Lepomis macrochirus*) under flow-through conditions
Document No	BW-85-10-1867, 10566.0985.6102.105
Guideline	US-EPA, Methods for acute tests with fish, macroinvertebrates and amphibians (1975)
GLP	Yes

Study summary and results:

The study was submitted and evaluated during first EU evaluation for Annex I inclusion of Bifenox. It was performed in compliance with Good Laboratory Practice (GLP) regulations and according to the current guideline at the time of study conduction. It is still considered acceptable for the renewal of Bifenox and the conduction of further vertebrate studies is considered inappropriate for animal welfare reasons.

The acute toxicity of Bifenox in bluegill sunfish was investigated under dynamic conditions for 96 hours. The test concentrations of Bifenox primarily solved in acetone were 0.18, 0.27, 0.42, 0.65 and 1.0 mg/L. The Bifenox concentrations in the test solutions were in average 27, 23, 22, 33 and 43 % of the nominal concentrations 1.0, 0.65, 0.42, 0.27 and 0.18 mg/L, respectively. Due to the degradation of Bifenox the evaluation of the results refers to average measured concentrations. The LC_{50} (96 h) of Bifenox for bluegill sunfish based on measured concentrations was determined to be > 0.27 mg/L. The NOEC in this study was 0.150 mg/L.

4.3.2 Short-term toxicity to aquatic invertebrates

4.3.2.1 Study 1 - CA 8.2.4.1 Acute toxicity to *Daphnia magna*

Study reference

Report	Anonymous (1985b)
Title	Acute toxicity of Bifenox to *Daphnia magna* under flow-through conditions
Document No	440169, BW-85-10-1871
Guideline	US-EPA, Methods for acute tests with fish, macroinvertebrates and amphibians (1975)
GLP	Yes

Study summary and results:

The study was submitted and evaluated during first EU evaluation for Annex I inclusion of Bifenox. It was performed in compliance with Good Laboratory Practice (GLP) regulations and according to US EPA Methods for the acute toxicity test with fish, macroinvertebrates, and amphibians (1975). It is still considered acceptable.

The acute toxicity of Bifenox in *Daphnia magna* was investigated under dynamic conditions for 48 hours. Nominal test concentrations of Bifenox primarily solved in acetone were 0.062, 0.12, 0.25, 0.50 and 1.0 mg/L. A control without addition of any further compounds and a solvent control were set up accordingly. The Bifenox concentrations in the test solutions were in average 0.018, 0.035, 0.074, 0.16 and 0.35 mg/L. Due to the degradation of Bifenox the evaluation of the results refers to average measured concentrations. The EC_{50} (48 h) of Bifenox for *Daphnia magna* based on measured concentrations was calculated to be > 0.66 mg/L, even higher than the range of test concentrations and exceeding the solubility of Bifenox. The NOEC with regard to behavioural abnormality in this study was 0.018 mg/L.

4.3.3 Algal growth inhibition tests

4.3.3.1 Study 1 - CA 8.2.6.1 Effects on growth of green algae

Study reference

Report	Anonymous (1998a)
Title	Technical Bifenox freshwater algal growth inhibition study and recovery phase (*Scenedesmus subspicatus*)
Document No	SA 98087, 603317
Guideline	OECD 201 (1984)
GLP	Yes

Study summary and results:

The test was submitted during the first EU evaluation for Annex I inclusion of Bifenox. It was conducted according to OECD guideline 201 (1984) and in compliance with Good Laboratory Practice (GLP) regulations. It fulfils the validity criteria according to the current guideline version (2011) and is considered valid and appropriate for the renewal of Bifenox and classification purposes. EC_x values have been re-calculated according to new data requirements. Since EC_{50}

values from this study were not provided during the first evaluation of Bifenox, but have been re-calculated with ToxRatPro Version 3.2.1 along with EC_{10}, EC_{20} and NOEC values, the new endpoints were used in the risk assessment for the re-evaluation of Bifenox as well as for classification purposes.

The toxicity of Bifenox to the freshwater alga *Scenedesmus subspicatus* under static conditions was determined during an exposure period of 72 hours and a following recovery period in untreated medium up to 6 days. Subcultures of *S. subspicatus* with an initial cell density of 2.0 to 2.1 x 10^4 cells/ml were exposed to nominal concentrations of 0.25, 1.0 and 1.5 µg a.s./L Bifenox solved in dimethylformamide (3 replicates per test concentration). A control without addition of any compound (6 replicates) and a solvent control (3 replicates) were tested in parallel. At test initiation, measured concentrations of Bifenox were comparable to the nominal values (93 to 104 %). At test termination Bifenox concentrations were reduced to 60, 84 and 93 % compared to nominal values. Exposure of *Scenedesmus subspicatus* to Bifenox for 72 hours resulted in a considerable inhibition in growth at concentrations between 0.25 to 1.5 µg a.s./L. During the recovery period it was demonstrated, that this inhibition is only transient and reversible. A full recovery was observed within 3 and 6 days and at all concentrations tested.

The re-calculated EC_{10}, EC_{20}, EC_{50} and NOEC values from this study are summarised below:

E_rC_{10} = 0.240 µg Bifenox/L

E_rC_{20} = 0.291 µg Bifenox/L

E_rC_{50} = 0.420 µg Bifenox/L

NOEC < 0.250 µg Bifenox/L

The complete calculations are provided in Document KCA 8.2.2.1/01.

4.3.3.2 Study 2 - CA 8.2.6.2 Effects on growth of an additional algal species

Study reference

Report	Anonymous (1999)
Title	Bifenox technical – Toxicity to the freshwater diatom, *Navicula pelliculosa*
Document No	604634, 10566.6577
Guideline	OECD 201 (1984)
GLP	Yes

Study summary and results:

The test was submitted during the first EU evaluation for Annex I inclusion of Bifenox. It was conducted according to OECD guideline 201 (1984) and in compliance with Good Laboratory Practice (GLP) regulations. It fulfils the validity criteria according to the current guideline version (2011) and is therefore still valid and appropriate for the renewal of Bifenox.

The toxicity of Bifenox to the freshwater diatom *Navicula pelliculosa* under static conditions was investigated for 72 hours in artificial culture medium. At test initiation, subcultures of *N. pelliculosa* corresponding to an initial cell density of 1.0 x 10^4 cells/ml were exposed to nominal concentrations of 0.05, 0.16, 0.51, 1.6, 5.2, 17 and 54 µg a.s./L Bifenox solved in DMF. A control containing culture medium without addition of any compound and a solvent control were tested in parallel. The mean measured concentrations of Bifenox, considering freshly prepared and aged solutions were 0.046, 0.16, 0.47, 1.6, 5.0, 15 and 49 µg a.s./L. Results are based on mean measured concentrations. A significant inhibition in growth was observed at concentrations of 0.47 µg/L and higher after 72 hours. The E_rC_{50} (72 h) was 38 µg a.s./L based on the growth rate, E_bC_{50} was 4.9 µg/L based on biomass. The NOEC (72 h) based on growth rate and biomass was 0.16 µg a.s./L.

The re-calculated EC_{10} and EC_{20} values from this study are summarised below:

E_rC_{10} = 3.803 µg Bifenox/L

E_rC_{20} = 8.161 µg Bifenox/L

The complete calculations are provided in Document KCA 8.2.2.1/01.

4.3.4 *Lemna* sp. growth inhibition test

4.3.4.1 Study 1 - CA 8.2.7 Effects on aquatic macrophytes

Study reference

Report	Anonymous (1998)
Title	Bifenox – Toxicity to the duckweed, *Lemna gibba*
Document No	603461, 98-10-7499
Guideline	FIFRA 122-2 and 123-3
GLP	Yes

Study summary and results:

The test was submitted during the first EU evaluation for Annex I inclusion of Bifenox. It was conducted according to EPA 122-2 and 123-3 and in compliance with Good Laboratory Practice (GLP) regulations, and is still considered valid and appropriate.

The toxicity of Bifenox to the duckweed *Lemna gibba* under semi-static conditions was investigated for 14 days in artificial culture medium. At test initiation, colonies of *Lemna gibba* were transferred to nominal concentrations of 0.63, 1.3, 2.5, 5.0 and 10.0 µg a.s./L Bifenox solved in DMF. A control containing culture medium without addition of any compound and a solvent control were tested in parallel. The test media were renewed on day 3, 6, 9 and 12. The mean measured concentrations of Bifenox, considering freshly prepared and aged solutions were 0.45, 1.1, 2.2, 4.5, and 9.6 µg a.s./L, indicating that Bifenox was stable under test conditions. Results are based on mean measured concentrations. Following the exposure of *Lemna gibba* to Bifenox a significant inhibition in growth was observed at concentrations of 1.1 µg/L and higher after 14 days. The E_rC_{50} (14 d) was 2.8 µg a.s./L and the NOEC was 0.45 µg a.s./L based on the number of fronds. The E_bC_{50} (14 d) was 2.1 µg a.s./L and the NOEC was < 0.45 µg a.s./L, based on biomass gain.

The re-calculated EC_{10} and EC_{20} values from this study are summarised below:

E_rC_{10} = 1.400 µg Bifenox/L

E_rC_{20} = 2.698 µg Bifenox/L

The complete calculations are provided in Document KCA 8.2.2.1/01.

4.3.4.2 Study 2 - CA 8.2.7 Effects on aquatic macrophytes

Study reference

Report	KCA 8.2.7/02, Anonymous (2016c)
Title	Macrophyte, growth Inhibition test – Bifenox (technical): sediment-free *Myriophyllum spicatum* toxicity test (OECD 238) semi-static conditions
Source	Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Auf dem Aberg 1, 57392 Schmallenberg, Germany
Report No	ADM-001/4-13/K, 90018357
Guidelines	OECD 238
GLP	Yes

Study summary and results:

The effects of the test item Bifenox technical (Batch no. 20120888) on the growth of the submersed rooted non-target aquatic plant species *Myriophyllum spicatum* were determined in a sediment-free system under sterile conditions over 14 days.

The plants were exposed to various test item concentrations under semi-static exposure conditions with renewal after 7 days. Based on a preliminary non-GLP range-finding test, 7 nominal concentration levels of the test item were chosen in a geometrical series with a spacing factor of 2.9: 0.080, 0.240, 0.710, 2.05, 5.95, 17.2 and 50.0 µg Bifenox/L. Acetone was used as a solvent.

Analytical evaluation of Bifenox was carried out at test start, after 7 days (both aged and freshly prepared test solutions) and at test end (14 days). On day 0 (start of the first 7 day exposure period) the measured concentrations were in the range of 93 to 111% of the nominal concentrations. At the end of the first 7 day exposure period the recovery ranged from 19.7 to 68.3% of the nominal concentrations. At start of the second exposure period (7 - 14 days) the measured concentrations were between 87.4 and 118% and at the end of the second exposure period between 23.6 and 60.0% of nominal concentrations. The test was evaluated based on the mean measured concentrations (0 - 14 days), i.e. the geometric means were calculated for the individual 7 day exposure periods and then the arithmetic mean value was drawn from the two geometric mean concentrations: Control, solvent control, 0.058, 0.197, 0.544, 1.47, 4.37, 10.2 and 22.1 µg Bifenox/L (0-14 days).

Fresh weight was the most sensitive growth rate parameter. For fresh weight, the EC_{50}-values for specific growth rate inhibition (E_rC50) was determined to be 0.661 µg a.s./L. The E_rC50 values for main shoot length and total shoot length were 1.36 and 1.89 µg a.s./L, respectively. The ErC50 for dry weight was 1.82 µg a.s./L and the E_rC50 for number of whorls was 1.64 µg a.s./L. For all growth rate parameters the NOEC was calculated to be 0.058 µg a.s./L. Fresh weight was also the most sensitive yield parameter. An E_yC50 value of 0.304 µg a.s./L was found. The E_yC50 values for dry weight, main shoot length and total shoot length were 0.611 µg a.s./L, 0.804 and 1.12 µg a.s./L. The E_yC50 for number of whorls was calculated to be 1.27 µg a.s./L. A NOEC of 0.058 µg a.s./L was determined for yield of main shoot length, fresh and dry weight and of 0.197 µg a.s./L for number of whorls. The NOEC for total shoot length was < 0.058 µg a.s./L, however, the E_yC10 was calculated to be 0.074 µg a.s./L.

In this study Bifenox was found to affect the growth of the non target aquatic plant species *Myriophyllum spicatum* after 14 days. The test was evaluated using the arithmetic mean drawn from the geometric mean concentrations of the two exposure periods. The test was evaluated based on the mean measured concentrations (0 - 14 days), i.e. the geometric means were calculated for the individual 7 day exposure periods and then the arithmetic mean value was drawn from the two geometric mean concentrations. Effective concentrations (EC_{10}, 20, 50) were calculated for the growth rate and yield of the measured parameters main shoot length, total shoot length, fresh and dry weights as well as number of whorls. Fresh weight was the most sensitive growth rate parameter. For fresh weight an E_rC_{50} value of 0.661 µg a.s./L was found. The E_rC50 values for main shoot length and total shoot length were 1.36 and 1.89 µg a.s./L, respectively. The E_rC_{50} for dry weight was 1.82 µg a.s./L and the E_rC_{50} for number of whorls was 1.64 µg a.s./L. For all growth rate parameters the NOEC was calculated to be 0.058 µg a.s./L. Fresh weight was also the most sensitive yield parameter. For fresh weight an E_yC_{50} value of 0.304 µg a.s./L was found, whereas the E_yC_{50} for dry weight was 0.611 µg a.s./L. The E_yC_{50} values for main shoot length and total shoot length were 0.804 and 1.12 µg a.s./L. The E_yC_{50} for number of whorls was calculated to be 1.27 µg a.s./L. A NOEC of 0.058 µg a.s./L was determined for yield of main shoot length, fresh and dry weight and of 0.197 µg a.s./L for number of whorls. The NOEC for total shoot length was determined to be < 0.058 µg a.s./L, however, the E_yC_{10} was calculated to be 0.074 µg a.s./L.

MATERIALS AND METHODS

Test Item

Designation	Bifenox (technical)
Characteristics	Solid, yellow tan crystals
Batch no.	20120888
Active ingredient / content	Bifenox / 98.811% according to CoA
Storage conditions	Store at room temperature 20 +/- 5°C, keep away from light and humidity

Stability	Stable at normal storage conditions in the dark
Solubility in water, trans-isomer	0.398 mg/L at 25°C
Expiry date	March 2017

Test System

Species	*Myriophyllum spicatum*, Haloragaceae, Dicotyledonous
Origin	Cultured at the Institut für Gewässerschutz MESOCOSM GmbH, Neu-Ulrichstein 5, 35315 Homberg (Ohm), Germany, originate from German Federal Environmental Agency, Dienstgebäude Marienfelde, Schichauweg 58, 12307 Berlin, Germany
Test water	Modified Andrews' medium containing sucrose, pH of the medium was set to pH 5.8 using HCl, medium was sterilised by autoclaving at 121°C for 20 min

Test Procedure and Conditions

Test duration	14 days, semi-static
Test vessels	Glass test tubes (inner diameter approximately 20 mm, length approximately 250 mm) with aluminium caps
Temperature	22.0 to 23.5°C
pH	4.83 - 5.17 in the fresh solution, 5.91 - 7.01 in the aged solution
Light regime	16 h light / 8 h dark, 100 - 150 µE × m^{-2} × s^{-1}

Study Design and Methods

In-life dates	29.04.2015 - 13.05.2015
Treatment	Nominal test concentrations were 0.080, 0.240, 0.710, 2.05, 5.95, 17.2 and 50.0 µg Bifenox/L. Five replicates per treatment and 10 replicates per control and solvent control (with acetone) were performed. Each replicate contained 1 plant shoot. The sensitivity of the test organism is routinely checked using 3,5-dichlorophenol.
Observations	Measurement of shoot length: At the beginning of the exposure, all test tubes were photographed for the determination of the shoot length of the individual plants. Using the photographic procedure, shoot length was determined after 7 days and at the end of the exposure, with length defined as length of the plant from the cut end to the leaf apices of the main shoot. At test end the plants were removed from the tubes and the length of the main shoot length, of the lateral branches and of the roots were measured with a ruler. Number of lateral branches and roots: At test end the plants were removed from the tubes and the number of lateral branches and roots was determined. Determination of fresh and dry weight: To obtain the respective mean biomass data for day 0 (test start), ten homogeneous additional test plants, prepared as described above, were transferred into test tubes containing tap water. After taking photos of these samples, the plants were removed from the test tubes, rinsed with distilled water and the fresh and dry weights were determined. Visual observations: Remarkable morphological changes like chlorosis, necrosis, deformations, discolorations etc. were documented.
Analytical verification	The concentrations of the test item in the water phase were assessed by chemical analysis of the active substance Bifenox using LC-MS/MS. Samples were taken from the test media preparation at the start of the exposure period (day 0 and 7) and from representative replicates per test and control at the end of the exposure periods (day 7 and 14).

Statistics	EC_{10}, EC_{20} and EC_{50} for growth rate and yield were calculated by probit analysis modified for continuous data or non-linear regression procedure according to OECD 238 if possible NOEC and LOEC values were calculated using ANOVA procedures for each treatment concentration with means separated by Williams' test, Step-down Jonckheere-Terpstra test procedure or Multiple Sequentially-rejective Welch-t-test after Bonferroni-Holm. All statistical analyses were conducted by the computer program ToxRat® Professional.

RESULTS AND DISCUSSION

Measured concentrations, analytical results

On day 0 (start of the first 7 day exposure period) the measured concentrations were in the range of 93 to 111% of the nominal concentrations. At the end of the first 7 day exposure period the recovery ranged from 19.7 to 68.3% of the nominal concentrations. At start of the second exposure period (7 - 14 days) the measured concentrations were between 87.4 and 118% and at the end of the second exposure period between 23.6 and 60.0% of nominal concentrations.

Biological data

The effects of the test material on the shoot length and biomass of *Myriophyllum spicatum* have been investigated over a 14-days period and the results are presented below.

Table 4.3.4-1 Effective concentrations ($EC_{10, 20, 50}$) and their 95%-confidence limits, NOEC and LOEC values of Bifenox technical after 14 days

	Shoot length	
	Inhibition of specific growth rate [µg a.s./L]	**Inhibition of yield [µg a.s./L]**
$E_{main\ shoot\ length}C_{10}$	0.069 (0.026 - 0.190)	0.047 (0.019 - 0.123)
$E_{main\ shoot\ length}C_{20}$	0.192 (0.073 - 0.517)	0.125 (0.051 - 0.315)
$E_{main\ shoot\ length}C_{50}$	1.36 (0.426 - 4.46)	0.804 (0.275 - 2.43)
$NOEC_{main\ shoot\ length}$	0.058	0.058
$LOEC_{main\ shoot\ length}$	0.197	0.197
$E_{total\ shoot\ length}C_{10}$	0.137 (0.091 - 0.190)	0.074 (0.027 - 0.209)
$E_{total\ shoot\ length}C_{20}$	0.338 (0.250 - 0.432)	0.188 (0.071 - 0.514)
$E_{total\ shoot\ length}C_{50}$	1.89 (1.60 - 2.21)	1.12 (0.346 - 3.72)
$NOEC_{total\ shoot\ length}$	0.058	<0.058
$LOEC_{total\ shoot\ length}$	0.197	≤ 0.058
	Fresh weight	
	Inhibition of specific growth rate [µg a.s./L]	**Inhibition of yield [µg a.s./L]**
$E_{fresh\ weight}C_{10}$	0.066 (0.028 - 0.156)	0.046 (0.016 - 0.126)
$E_{fresh\ weight}C_{20}$	0.145 (0.064 - 0.337)	0.087 (0.033 - 0.236)
$E_{fresh\ weight}C_{50}$	0.661 (0.247 - 1.81)	0.304 (0.095 - 0.999)
$NOEC_{fresh\ weight}$	0.058	0.058
$LOEC_{fresh\ weight}$	0.197	0.197
	Dry weight	
	Inhibition of specific growth rate [µg a.s./L]	**Inhibition of yield [µg a.s./L]**
$E_{dry\ weight}C_{10}$	0.025 (0.004 - 0.178)	0.013 (0.001 - 0.119)
$E_{dry\ weight}C_{20}$	0.109 (0.017 - 0.746)	0.049 (0.006 - 0.417)
$E_{dry\ weight}C_{50}$	1.82 (0.187 - 18.1)	0.611 (0.052 - 7.86)
$NOEC_{dry\ weight}$	0.058	0.058
$LOEC_{dry\ weight}$	0.197	0.197
	Number of whorls	
	Inhibition of specific growth rate [µg a.s./L]	**Inhibition of yield [µg a.s./L]**

EC_{10}	0.302 (0.149 - 0.629)	0.221 (0.111 - 0.453)
EC_{20}	0.540 (0.270 - 1.10)	0.403 (0.204 - 0.809)
EC_{50}	1.64 (0.702 - 3.85)	1.27 (0.553 - 2.92)
NOEC	0.058	0.197
LOEC	0.197	0.544

Additional information: Effects on roots

There were concentration-dependent effects on the number and total root length. The growth of the roots was inhibited at concentrations higher than 0.058 µg Bifenox/L compared with the control and solvent control plants. As noted in OECD 238, the inclusion of root endpoints for evaluation of effects is questionable in the no-sediment test for *Myriophyllum*. This is due to the fact that sucrose and light (roots are clearly exposed to light during the test) may have an influence on auxin (plant growth hormone) transport carriers, and that some chemicals may have an auxin-type mode of action. Therefore, the parameter root development does not provide reliable results, and therefore this information is only presented as additional information

Additional information: Visual observations

The appearances of the plants at the two lowest test concentrations were comparable to that of the control plants. With increasing test concentrations the leafs of the upper part of the plants became smaller and darker green than the other part of the plants (at 0.544 and 1.47 µg a.s./L one third of the plant, at 4.37 µg/L half of the plant and at the two highest concentrations the whole plant). Reddish heads were observed at 1.47 µg a.s./L and at the two highest test concentrations.

CONCLUSIONS

In this study Bifenox was found to affect the growth of the non target aquatic plant species *Myriophyllum spicatum* after 14 days. The test was evaluated using the arithmetic mean drawn from the geometric mean concentrations of the two exposure periods. The test was evaluated based on the mean measured concentrations (0 - 14 days), i.e. the geometric means were calculated for the individual 7 day exposure periods and then the arithmetic mean value was drawn from the two geometric mean concentrations. Effective concentrations ($EC_{10, 20, 50}$) were calculated for the growth rate and yield of the measured parameters main shoot length, total shoot length, fresh and dry weights as well as number of whorls. Fresh weight was the most sensitive growth rate parameter. For fresh weight an E_rC_{50} value of 0.661 µg a.s./L was found. The E_rC_{50} values for main shoot length and total shoot length were 1.36 and 1.89 µg a.s./L, respectively. The E_rC_{50} for dry weight was 1.82 µg a.s./L and the E_rC_{50} for number of whorls was 1.64 µg a.s./L. For all growth rate parameters the NOEC was calculated to be 0.058 µg a.s./L. Fresh weight was also the most sensitive yield parameter. For fresh weight an EyC_{50} value of 0.304 µg a.s./L was found, whereas the E_yC_{50} for dry weight was 0.611 µg a.s./L. The E_yC_{50} values for main shoot length and total shoot length were 0.804 and 1.12 µg a.s./L. The E_yC_{50} for number of whorls was calculated to be 1.27 µg a.s./L. A NOEC of 0.058 µg a.s./L was determined for yield of main shoot length, fresh and dry weight and of 0.197 µg a.s./L for number of whorls. The NOEC for total shoot length was determined to be < 0.058 µg a.s./L, however, the E_yC_{10} was calculated to be 0.074 µg a.s./L

4.3.4.3 Study 3 - CA 8.2.7 Effects on aquatic macrophytes

Study reference

Report	KCA 8.2.7/07, Anonymous (2016h)
Title	Macrophyte, water-sediment toxicity test (OECD 239) – Bifenox (technical): Semi-static Water-Sediment *Myriophyllum spicatum* Toxicity Test – testing for recovery of growth
Source	Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Auf dem Aberg 1, 57392 Schmallenberg, Germany
Report No	ADM-001/4-12/K, 90019665
Guidelines	OECD 239 (2014)
GLP	Yes

Study summary and results:

The effects of the test item Bifenox technical on the growth of the submersed rooted non target aquatic plant species *Myriophyllum spicatum* were determined in a water-sediment system under sterile conditions over 14 days, followed by a subsequent recovery phase of 21 days.

The plants were exposed to various test item concentrations under semi-static exposure conditions with renewal after 7 days. Based on a preliminary non-GLP range-finding test, 5 nominal concentration levels of the test item were chosen in a geometrical series with a spacing factor of 3.16: 0.150, 0.475, 1.50, 4.75 and 15.0 µg Bifenox (technical)/L. Acetone was used as a solvent. The 14-day growth test was followed by a subsequent recovery phase

of up to 21 days with growth medium renewal after 14 days.

Analytical evaluation of Bifenox was carried out at test start and at the end of the 7-day exposure periods using LC-MS/MS (LOQ = 0.05 µg/L). On day 0 (start of the first 7 day exposure period) the measured concentrations were in the range of 96.8 to 129% of the nominal concentrations. At the end of the first 7 day exposure period the concentrations were below the LOQ in the lowest treatment and ranged between 9.0 and 17.1% of nominal in the other treatments. At start of the second exposure period (7 - 14 days) the measured concentrations were between 81.2 to 92.0% of the nominal concentrations. At the end of the second 7-day exposure period, Bifenox concentrations were below the LOQ in the two lowest treatments and ranged between 9.08 and 29.6% of nominal in the other treatments. Since the test item concentrations declined over the test period, the average exposure concentrations were calculated as time-weighted mean. The mean measured concentrations were 0.064, 0.173, 0.559, 1.90 and 6.84 µg Bifenox/L.

Since the measured concentrations of the test item in the water phase were below 80% of the nominal concentrations at test end, concentrations in the continuously exposed sediment were determined during the test, according to OECD 239. At test start Bifenox was only found in concentrations above LOQ in sediment of the highest treatment (0.840 µg/kg sediment dw). After one day, Bifenox was above LOQ in the two highest test concentrations (0.899 and 3.51 µg/kg sediment dw) and after seven days in the three highest treatments (0.845, 3.25 and 8.26 µg/kg sediment dw). After 14 days 2.12 and 6.54 µg/kg sediment dw were analysed in sediment of the two highest test concentrations.

MATERIALS AND METHODS

Test Item

Designation	Bifenox (technical)
Characteristics	Solid, yellow tan crystals
Batch no.	20120888

Active ingredient / content	Bifenox / 98.811% according to CoA
Storage conditions	Store at room temperature 20 +/- 5°C, keep away from light and humidity
Stability	Stable at normal storage conditions in the dark
Solubility in water	< 0.1 mg/L at 20°C
Expiry date	March 2017

Test System

Species	*Myriophyllum spicatum*, Haloragaceae, Dicotyledonous
Origin	The sterile plants originate from the German Federal Environmental Agency, Dienstgebäude Marienfelde, Schichauweg 58, 12307 Berlin, Germany.
Test water	Smart & Barko medium was used as liquid growth medium. At test initiation, the pH of the medium was adjusted to a level of 7.5 - 8.0 to allow optimum plant growth (using HCl 37%).
Test sediment	Sediment based on the artificial soil used in OECD Guideline 219 was used

Test Procedure and Conditions

Test duration	Exposure phase: 14 days, recovery phase: 21 days
Test vessels	2 L glass beakers (approx. 24 cm high and 11.2 cm inner diameter) as replicate housing. Small plastic plant pots (approx. 9.5 cm inner diameter, outer diameter 11 cm, and 8.5 cm high, commercially available) were used for potting the plants into the sediment. The exposed sediment surface of 70.9 cm^2 represented about 72% of the cross-sectional area of the glass test vessels (98.5 m^2) in which the pots were individually placed. Plant pots were prepared with a filter paper in the bottom of the pot to stop sediment loss. The nutrient supplemented sediment was transferred into the plant pot (about 6 cm high, volume ca. 420 mL) followed by a fine layer (approx. 2 mm) of coarse quartz sand in order to reduce suspension of sediment into the water. The minimum overlaying water depth was 12 cm above the top of the sediment. The ratio of sediment surface area/volume (approximately 0.169 cm^2/mL) to the ratio of water surface area/volume (approximately 0.058 cm^2/mL) was around three.
Temperature	20.5 - 22.0°C
pH	7.65 - 7.60 at test start, 9.96 - 10.22 for the individual control and solvent control vessels
Light regime	16 h light / 8 h dark, 120.2 - 158.9 $\mu E \times m^{-2} \times s^{-1}$

Study Design and Methods

In-life dates	05.04.2016 - 19.04.2016
Treatment	Nominal test concentrations were 0.150, 0.475, 1.50, 4.75 and 15.0 µg Bifenox (technical)/L. Five replicates per treatment and 10 replicates per control and solvent control (with acetone) were used for the growth test. For the assessment of recovery, five replicate test vessels were additionally prepared for each treatment group and ten replicates for the additional control group with each replicate consisting of three plants. The sensitivity of the test organism is routinely checked using 3,5-dichlorophenol.

Observations	The growth parameters: Main shoot length, length of lateral branches, development of fresh and dry weight were recorded. Main shoot length and length of lateral branches parameters were used to calculate the total shoot length, representing the total plant biomass. Length measurements of main shoots, lateral branches and roots: Shoot length (cm) was determined in-situ on days 0, 7 and 14, from the level of the sediment, using a small plastic ruler inserted into the test vessel and standing against the straightened plant. If side shoots were present, their numbers and length were measured using a ruler. Fresh and dry weight: Fresh weight was assessed using the plant shoot above sediment. The plants were carefully cut at sediment level. The plants were weighed after the remaining medium was carefully blotted off. Dry weight (mg) was determined after oven drying at 60°C for 48 h. At test end (day 14) the roots (sediment covered part of the plant) were carefully removed from the pot, washed with water and a visual assessment of the roots was made according to the following legend: 1 roots absent, 2 few roots, 3 moderate root development, 4 very good root development - similar to controls Visual observations: Remarkable morphological changes like chlorosis, necrosis, deformations, discolorations etc. were documented on days 7 and 14.
Analytical verification	The concentrations of the test item in the water phase were assessed by chemical analysis of the active substance Bifenox using LC-MS/MS (LOQ in test medium = 0.05 µg a.s./L, 0.77 µg/kg dry sediment). Samples were taken at start (1 hour after application of the test medium, according to OECD 239), after 1 day and at the end of each 7-day exposure period of the 14-day semi-static test. At start of the exposure periods (fresh media), samples (2 × 20 mL) were taken from the additionally prepared vessels. At the end of the exposure periods (aged media) samples were taken from representative replicates per test concentration and controls. Sediment samples were taken from additionally prepared vessels at test start (1 hour after application of the test medium, according to OECD 239, after 1 and after 7 days. At test termination, sediment was sampled from representative replicates of all treatments and controls.
Statistics	EC_{10}, EC_{20} and EC_{50} for growth rate and yield were calculated by non-linear regression procedures, e.g. a 4-parametric logistic cumulative normal distribution function (yield, total shoot length) according to Bruce and Versteeg (1992), were fitted to the data. Subsequently, other non-linear regression models provided by the computer programme ToxRat Professional® were used. Since for growth rate of total shoot length, fresh and dry weight as well as for yield fresh and dry weight there were significant lacks of fit with all models, the EC_{10}, EC_{20} and EC_{50} values of these parameters were calculated by linear regression (Probit analysis, based on normal distribution of the data (sigmoid normal), modified for continuous data. Individual replicate responses were used for the regression analysis.

II. RESULTS AND DISCUSSION

Measured concentrations, analytical results

Concentrations in test media

At test start (one hour after test initiation), measured Bifenox concentrations in test media were 0.193, 0.509, 1.45, 4.95 and 16.6 µg Bifenox/L (i.e. 96.8 - 129% of nominal). After one day, the Bifenox levels declined to concentrations between 56.8 and 71.4% of nominal. At the end of the first exposure period, after 7 days, Bifenox concentrations were below the LOQ in the lowest treatment and ranged between 9.0 and 17.1% of nominal in the other treatments. At start of the second exposure period (one hour after medium renewal), measured Bifenox concentrations in test media were 0.125, 0.401, 1.22, 4.15 and 13.8 µg Bifenox/L (i.e. 81.2 - 92.0% of nominal). After one day, Bifenox levels declined to concentrations between 65.0 and 81.9% of nominal. At the end of the second 7-day exposure period, Bifenox concentrations were below

the LOQ in the two lowest treatments and ranged between 9.08 and 29.6% of nominal in the other treatments. Since the test item concentrations declined over the test period and between the different intervals of the medium analysis, the average exposure concentrations were calculated as time-weighted mean. For concentrations below the LOQ one-half the LOQ was used for calculation.

Concentrations in sediment

Since the measured concentrations of the test item in the water phase were below 80% of the nominal concentrations at test end, concentrations in the continuously exposed sediment were determined during the test, according to OECD 239. At one day after test start Bifenox concentrations in the three lowest treatments were below the LOQ. On day 7 at the end of the first exposure period, Bifenox concentrations in the three highest treatments were 0.845, 3.25 and 8.26 µg Bifenox/L. In the two lowest treatments the Bifenox concentrations were below the LOQ. At the end of the second exposure period (day 14) only in the two highest treatments the test item concentrations were above the LOQ (2.12 and 6.56 µg Bifenox/L).

Biological data

Growth inhibition test

The effects of the test material on the shoot length and biomass of *Myriophyllum spicatum* have been investigated over a 14-days exposure period and the results are presented below.

Table 4.3.4-2 Effective concentrations ($EC_{10, 20, 50}$) and their 95%-confidence limits, NOEC and LOEC values of Bifenox technical after 14 days

	Shoot length	
	Inhibition of specific growth rate [µg a.s./L]	**Inhibition of yield [µg a.s./L]**
$E_{total\ shoot\ length}C_{10}$	0.098 (0.066 - 0.146)	0.063 (0.028 - 102)
$E_{total\ shoot\ length}C_{20}$	0.185 (0.126 - 0.276)	0.119 (0.065 - 0.169)
$E_{total\ shoot\ length}C_{50}$	0.629 (0.388 - 1.01)	0.346 (0.246 - 0.449)
$NOEC_{total\ shoot\ length}$	0.064	0.064
$LOEC_{total\ shoot\ length}$	0.173	0.173
	Fresh weight	
	Inhibition of specific growth rate [µg a.s./L]	**Inhibition of yield [µg a.s./L]**
$E_{fresh\ weight}C_{10}$	0.057* (0.034 - 0.082)	0.016** (0.003 - 0.040)
$E_{fresh\ weight}C_{20}$	0.119 (0.082 - 0.157)	0.049* (0.014 - 0.092)
$E_{fresh\ weight}C_{50}$	0.488 (0.398 - 0.599)	0.252 (0.157 - 0.352)
$NOEC_{fresh\ weight}$	< 0.064	< 0.064
$LOEC_{fresh\ weight}$	≤ 0.064	≤ 0.064
	Dry weight	
	Inhibition of specific growth rate [µg a.s./L]	**Inhibition of yield [µg a.s./L]**
$E_{dry\ weight}C_{10}$	0.082 (0.030 - 0.153)	0.015* (0.002 - 0.045)
$E_{dry\ weight}C_{20}$	0.224 (0.113 - 0.354)	0.067 (0.015 - 0.146)
$E_{dry\ weight}C_{50}$	1.52 (1.07 - 2.26)	0.646 (0.359 - 0.953)
$NOEC_{dry\ weight}$	< 0.064	< 0.064
$LOEC_{dry\ weight}$	≤ 0.064	≤ 0.064

Additional information: Visual observations

At the end of the first 7-day exposure period no abnormalities were observed at concentrations up to and including 1.90 µg Bifenox/L. At the highest test item concentration, slightly brownish leaves were observed. At the end of the second 7-day exposure period partly white and chlorotic leaves were observed at 0.559 µg Bifenox/L. At 1.90 µg Bifenox/L plants were almost completely white and chlorotic and the tip of the shoot was covered with an algal film. At the highest test item concentration leaves appeared slightly brownish and plants were completely white and chlorotic. The plants appeared to be fragile and were covered with an algal film.

Recovery test

The recovery effects on the shoot length and biomass of *Myriophyllum spicatum* have been investigated over a 21-days recovery period after 14-days exposure period and the results are presented below.

Table 4.3.4-3 Effective concentrations ($EC_{10, 20, 50}$) and their 95%-confidence limits, NOEC and LOEC values of Bifenox technical during a 21-day recovery

	Shoot length	
	14-day recovery [µg a.s./L]	**21-day recovery [µg a.s./L]**
$E_{total\ shoot\ length}C_{10}$	0.329 (0.130 - 0.546)	0.172 (0.041 - 0.353)
$E_{total\ shoot\ length}C_{20}$	0.634 (0.333 - 0.914)	0.451 (0.174 - 0.762)
$E_{total\ shoot\ length}C_{50}$	1.94 (1.39 - 2.51)	2.34 (1.46 - 3.25)
$NOEC_{total\ shoot\ length}$	0.173	0.173
$LOEC_{total\ shoot\ length}$	0.559	0.559
	Fresh weight	
	Inhibition of specific growth rate [µg a.s./L]	**Inhibition of yield [µg a.s./L]**
$E_{fresh\ weight}C_{10}$	4.09 (n.d.)	0.195 (0.110 - 0.286)
$E_{fresh\ weight}C_{20}$	4.53 (n.d.)	0.368 (0.244 - 0.494)
$E_{fresh\ weight}C_{50}$	5.51 (n.d.)	1.25 (0.994 - 1.57)
$NOEC_{fresh\ weight}$	1.90	0.064
$LOEC_{fresh\ weight}$	6.84	0.173
	Dry weight	
	Inhibition of specific growth rate [µg a.s./L]	**Inhibition of yield [µg a.s./L]**
$E_{dry\ weight}C_{10}$	2.25 (1.53 - 2.84)	0.256 (0.179 - 0.334)
$E_{dry\ weight}C_{20}$	2.91 (2.16 - 3.49)	0.452 (0.348 - 0.553)
$E_{dry\ weight}C_{50}$	4.76 (4.10 - 5.33)	1.34 (1.15 - 1.56)
$NOEC_{dry\ weight}$	1.90	0.173
$LOEC_{dry\ weight}$	6.84	0.559

Additional information: Visual observations

After 7, 14 and 21 days recovery no abnormalities on plants were observed in the solvent control and the pre-exposure concentrations of 0.064 and 0.173 µg Bifenox/L. After 7 days of recovery leaves appeared partly white and chlorotic and side shoots and shoot tips were partly green-reddish at the pre-exposure concentration of 0.559 µg Bifenox/L. After 14 and 21 days leaves appeared green again, but shoot tips were still partly green-reddish at this pre-exposure concentration. The plants pre-exposed to 1.90 µg Bifenox/L were partly white and chlorotic after 7 days of recovery. Side shoots and shoot tips were partly green-reddish and leaves were covered with a thin algal film. Seven and fourteen days later after 14 days and 21 days of recovery, respectively, plants were green again, but leaves were still covered with a thin algal film and side shoots and shoot tips remained partly green-reddish. At the highest pre-exposure concentration of 6.84 µg Bifenox/L plant were completely white and chlorotic and appeared fragile with algal film. Seven and 14 days later after 14 and 21 days recovery, respectively plants were partly green again, but still fragile with algal film. New side shoots were green reddish, but without algal film and stable.

CONCLUSIONS

The effects of the test item Bifenox technical on the growth of the submersed rooted non target aquatic plant species *Myriophyllum spicatum* were determined in a water-sediment system under sterile conditions over 14 days, followed by a subsequent recovery phase of 21 days. The plants were exposed to various test item concentrations under semi-static exposure conditions with renewal after 7 days. Based on a preliminary non-GLP range-finding test, 5 nominal concentration levels of the test item were chosen in a geometrical series with a spacing factor of 3.16: 0.150, 0.475, 1.50, 4.75 and 15.0 µg Bifenox/L. Acetone was used as a solvent. The 14-day growth test was followed by a subsequent recovery phase of up to 21 days with growth medium renewal after 14 days.

Growth inhibition test

Effective concentrations ($EC_{10, 20, 50}$) were calculated for the growth rate and yield of the measured parameters main shoot length, total shoot length, fresh and dry weights. For growth rate, fresh weight was the most sensitive parameter. The E_rC_{50} for fresh weight was 0.488 µg Bifenox//L. For dry weight an E_rC_{50} value of 1.52 µg Bifenox//L was found. The E_rC_{50} value for total shoot length was 0.629 µg/L. The NOEC values for growth rate of the dry and fresh weight were calculated to be < 0.064 µg Bifenox//L. For yield fresh weight was also the most sensitive parameter. The E_YC_{50} for fresh weight was 0.252 µg Bifenox//L. For dry weight an E_YC_{50} value of 0.646 µg/L was found. The E_YC_{50} value for total shoot length was 0.346 µg Bifenox/ /L. The NOEC for fresh weight and dry weight of the yield parameter was calculated to be < 0.064 µg Bifenox//L.

Recovery test

Complete recovery of the plants pre-exposed up to the highest concentration of 6.84 µg Bifenox/L could be demonstrated based on the sectional growth rates of total shoot length after a recovery period of 14 days. The NOEC was found to be ≥ 6.84 µg Bifenox/L (mean measured) compared to 0.064 µg Bifenox/L derived for the exposure period. Based on the overall 0 - 21 day growth rates of the other parameters fresh and dry weight the potential for recovery was demonstrated for the plants pre-exposed up to 1.90 µg Bifenox/L and the NOEC values were 1.90 µg Bifenox/L (mean measured concentrations of the exposure period) compared to < 0.064 µg Bifenox/L derived for the exposure period. Effective concentrations ($EC_{10, 20, 50}$ and NOEC) were calculated for growth rate and yield of the measured parameters total shoot length, fresh and dry weights. Additionally, the sectional growth rates of total shoot length were evaluated. After a 14-day recovery an E_yC_{50} value of 1.94 µg Bifenox/L for yield total shoot length was determined. After a recovery period of 21 days the E_yC_{50} increased to 2.34 µg Bifenox/L. For fresh weight recovery, an EC_{50} value for growth rate of 5.51 µg Bifenox/L and for yield of 1.25 µg Bifenox/L were determined for the 21-day recovery period. For dry weight recovery, an EC_{50} value for growth rate of 4.76 µg Bifenox/L and for yield of 1.34 µg Bifenox/L were determined for the 21-day recovery period.

4.4 Chronic toxicity

4.4.1 Fish early-life stage (FELS) toxicity test

Not relevant.

4.4.2 Fish short-term toxicity test on embryo and sac-fry stages

Not relevant.

4.4.3 Aquatic Toxicity – Fish, juvenile growth test

4.4.3.1 Study 1 - CA 8.2.2.1 Fish early life stage toxicity test

Study reference

Report	Anonymous (1991)
Title	The prolonged toxicity of Bifenox to rainbow trout (*Oncorhynchus mykiss*)
Document No	282/113, 426191
Guideline	OECD 204 (1984)
GLP	Yes

Study summary and results:

The study was submitted and evaluated during first EU evaluation for Annex I inclusion of Bifenox. The study was performed in compliance with Good Laboratory Practice (GLP) regulations and according to the current guideline at the time of study conduction (OECD 204). It is still considered acceptable and the conduction of further vertebrate studies is considered inappropriate for animal welfare reasons.

The toxicity of Bifenox in rainbow trout was investigated under dynamic conditions for 21 days. Bifenox (solved in 1% Tween 80-acetone) was continuously dosed to the water flow to final concentrations of 0.0032, 0.010, 0.032, 0.100 and 0.320 mg/L. A control without addition of any further compounds and a solvent control were set up accordingly. The Bifenox concentrations in the test solutions were in the range of 75 % to 91 % of the nominal concentrations. Due to the decrease of Bifenox in the test solution the, evaluation of the results refers to average measured concentrations. No mortality was observed up to a Bifenox concentration of 0.024 mg/L. The LC_{50} for Rainbow trout following an exposure to Bifenox for 21 days was 0.12 mg/L, based on measured concentrations. The NOEC was 0.0091 mg/L, also based on measured concentrations.

The re-calculation of LC_{10} and LC_{20} values from this study was not possible due to incomplete raw data.

4.4.3.2 Study 2 - CA 8.2.2.1 Fish early life stage toxicity test

Study reference

Report	Anonymous (1981)
Title	Dynamic toxicity of Bifenox to bluegill sunfish (*Lepomis macrochirus*)
Document No	27115, 440247
Guideline	US-EPA, Methods for acute tests with fish, macroinvertebrates and amphibians (1975)
GLP	Yes

Study summary and results:

The study was submitted and evaluated during first EU evaluation for Annex I inclusion of Bifenox. The study was performed in compliance with Good Laboratory Practice (GLP) regulations and according to the current guideline at the

time of study conduction (US EPA 660375009, 1975). It is still considered acceptable and the conduction of further vertebrate studies is considered inappropriate for animal welfare reasons.

The toxicity of Bifenox in bluegill sunfish was investigated under dynamic conditions for 14 days. The fish loading was 0.03 g body weight/L (20 fish/test vessel with a mean body weight of 0.72 in 30 L of test medium with renewal of 16 tank volumes per day). Bifenox in acetone was continuously dosed to the water flow to final concentrations of 0.052, 0.10, 0.22, 0.49 and 1.00 mg/L. A control without addition of any further compounds was set up accordingly. The Bifenox concentrations in the test solutions were ranging between 90 and 130 % of the nominal concentrations. Evaluation of the results refers to average measured concentrations. No mortality was observed in the control and in the treatment group up to a Bifenox concentration of 0.13 mg/L. The LC_{50} for bluegill sunfish following an exposure to Bifenox for 14 days was 0.46 mg/L, based on measured concentrations. The NOEC was 0.13 mg/L.

The **re-calculated LC_{10} and LC_{20} values** from this study are summarised below:

LC_{10} = 0.265 mg Bifenox/L

LC_{20} = 0.320 mg Bifenox/L

The complete calculations are provided in Document KCA 8.2.2.1/01.

4.4.4 Chronic toxicity to aquatic invertebrates

4.4.4.1 Study 1 - CA 8.2.5.1 Reproductive and development toxicity to *Daphnia magna*

Study reference

Report	Anonymous (1999)
Title	Technical Bifenox *Daphnia magna* reproduction test
Document No	SA 98275, 603539
Guideline	OECD 211 (1997)
GLP	Yes

Study summary and results:

The study was submitted and evaluated for Annex I inclusion of Bifenox. It was conducted in compliance with OECD guideline 211 and Good Laboratory Practice (GLP) regulations. Thus, it is still in compliance with the current requirements and is therefore still valid and appropriate.

The reproductive toxicity of Bifenox technical on *Daphnia magna* was investigated for 18 days after a 3-days exposure period. Young *D. magna* were exposed to a solvent control and to nominal Bifenox concentrations of 0 (control), 2.5, 10.0 and 15.0 µg/L under static conditions for 72 hours. The initially measured concentrations of Bifenox were close to the nominal values (94 - 104 % recovery), Following the 72 hours exposure period, the concentrations decreased to 40 to 42 % of the nominal concentrations. All parent animals survived the 72-hours exposure period to Bifenox and were transferred into untreated medium. No substance related immobilisation and no reduction in reproduction of *Daphnia magna* was observed at any concentrations. The overall NOEC (21 days) was 15.0 µg/L.

EC_{10} and EC_{20} values cannot be re-calculated due to incomplete data.

4.4.4.2 Study 2 - CA 8.2.5.1 Reproductive and development toxicity to *Daphnia magna*

Study reference

Report	Anonymous (1990)
Title	21-Day chronic static renewal toxicity of Bifenox to *Daphnia magna*
Document No	38461, 441956
Guideline	OECD 211 (1997)
GLP	Yes

Study summary and results:

The study was submitted and evaluated for Annex I inclusion of Bifenox. It was conducted in compliance with OECD guideline 211 and Good Laboratory Practice (GLP) regulations. Thus, it is still in compliance with the current requirements and are therefore still valid and appropriate.

The reproductive toxicity of Bifenox technical on *Daphnia magna* was investigated during a 21-days exposure period. *D. magna* were exposed to a solvent control and to nominal Bifenox concentrations of 0 (control), 0.12, 0.24, 0.95 and 1.9 µg/L under semi-static conditions for 21 days. The mean measured concentrations of Bifenox, considering freshly prepared and aged solutions were 0.066, 0.15, 0.33, 0.72 and 1.4 µg/L. Due to the degradation of Bifenox during the study period the evaluation of the results refers to average measured concentrations. At termination of the study no immobilisation was observed at concentrations of 0.33 µg a.s./L and below. At levels of 0.72 and 1.4 µg a.s./L a parental immobilisation of 8 and 4 % (statistically not significant) was observed. The reproductive performance was not inhibited at concentrations of 0.33 µg a.s./L and below. Based on mean measured Bifenox concentrations the NOEC (21 d) for *Daphnia magna* was 0.15 µg a.s./L, due to the reduction in body length. The NOAEC (21 d), based on the reproduction rate was 0.33 µg/L. The EC_{50} (21 d) was > 1.4 µg a.s./L.

The re-calculated EC_{10} and EC_{20} values from this study are summarised below:

EC_{10} = 0.296 µg Bifenox/L

EC_{20} = 0.551 µg Bifenox/L

The complete calculations are provided in Document KCA 8.2.2.1/01.

4.4.5 Chronic toxicity to algae or aquatic plants

Please refer to Point 4.3.3.

4.5 Acute and/or chronic toxicity to other aquatic organisms

4.5.1 Study 1 - CA 8.2.5.3 Development and emergence in *Chironomus riparius*

Study reference

Report	Anonymous (1996)
Title	Bifenox toxicity to the sediment dwelling chironomid larvae (*Chironomus riparius*) under static conditions
Document No	SA 95480, 601283
Guideline	BBA Guideline Proposal: Effects of plant protection products on the development of sediment dwelling larvae of *Chironomus riparius* in a water-sediment system (1995)
GLP	Yes

Study summary and results:

The study was submitted and evaluated for Annex I inclusion of Bifenox. It was conducted according to a proposal for a BBA guideline (1995) and in compliance with Good Laboratory Practice (GLP) regulations. It is still in compliance with the current requirements and are therefore still valid and appropriate.

The toxicity of Bifenox to larvae of the sediment dwelling midge *Chironomus riparius* was investigated in a static water-sediment system for 28 days. At test start, Bifenox solved in acetone was added to the test units in amounts to achieve initial nominal water concentrations of 1.0, 1.5, 12.5, 15, and 125 µg a.s./L. A control without addition of any further compounds and a solvent control were set up accordingly. Bifenox concentrations one hour after test start were confirmed to be 103 to 128 % of the nominal concentrations. On day 7 and 28 Bifenox levels in the water were below the limit of quantification. Following the exposure of first instar larvae of *Chironomus riparius* to Bifenox, the NOEC was 15 µg/L. The LOEC was 125 µg/L since at this concentration the rate of emergence was significantly reduced.

The re-calculation of EC_{10} and EC_{20} values from this study is not possible due to number of number of test concentrations with analytical verification and spacing.