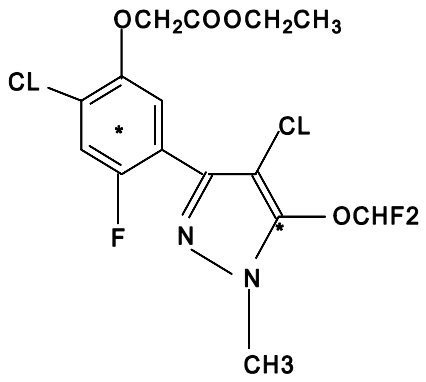
ANNEX B

Pyraflufen-ethyl

B.5 Toxicology and metabolism



B.5.1 Absorption, distribution, excretion and metabolism (toxicokinetics) (Annex IIA 5.1)

*: position of radiolabeling : radiolabeling with ^{14}C was on the phenyl-U- or on the pyrazole-5-.

Findings :
Absorption :
Absorption is rapid, but incomplete and dose dependent. During the first 48 h after single oral low dose administration, radioactivity excreted in bile was 36.09% and in urine 19.66%, from which an absorption from the gastrointestinal tract of 55.75% can be calculated (table B.5.1.3-7).
Increasing the dose by a factor of 100 reduces urinary excretion (2.38-3.66%) with a concommitant increase of fecal excretion, mainly unchanged pyraflufen-ethyl, reaching 90%. This suggests that bioavailability decreases with increasing doses (table B.5.1.3-1). The results are independent of labelling position.

The area under blood or plasma concentration versus time curve (AUC), the time required (Tmax) to reach peak concentration of radioactivity in plasma and blood, the maximum concentration (Cmax) reached in blood and plasma were calculated and summarized in table B.5.1.3-2.
- After oral low dose administration, maximal blood and plasma concentration was reached in male and female rats after 3-5.4 h. Blood concentration was lower than plasma concentration indicating that major parts of radioactivity did not penetrate into cellular blood constituents. While the Cmax were comparable for male and female rats, the AUC values were lower for females and suggest a higher bioavailability for males.
- After multiple dose administration, Cmax, Tmax and T1/2 were not significantly affected. While AUC value in plasma was reduced, blood AUC value was increased as compared with single dose administration and may suggest an accumulation of the compound in blood.
- After high dose administration, peak concentration was somewhat delayed (4.2-7.8 h); Cmax and AUC values increased but not proportional to the dose indicating that the process of absorption from the gastrointestinal tract saturated at the high dose level. AUC values and T1/2 were higher for males than for females and are indicative of higher and/or longer sustained concentrations in male plasma/blood than in females.

Radioactivity was eliminated rapidly from blood and plasma and was near the detection limit after 96 h. Regardless to the sex of animals and dose administered, radioactivity concentrations in blood and plasma decline with a single exponential decline.

Table B.5.1.3 -1: Plasma and blood concentrations of radioactivity after a single or repeated oral low or high dose of pyraflufen-ethyl (pyrazole-5- ^{14}C) (Motoba, 1996a)

	Radioactivity in plasma or blood (µg equivalents to pyraflufen-ethyl/g)							
Dose	**1 x 5 /15 x 5 mg/kg bw**				**500 mg/kg bw**			
Time (h)	_		_		_		_	
Tissue	plasma	blood	plasma	blood	plasma	blood	plasma	blood
1	0.998/ 2.15	0.496/ 0.778	0.830	0.417	17.2	8.5	21.4	11.3
3	**2.514/ 2.64**	**1.060/ 0.896**	**2.673**	**1.275**	71.7	31	**99.7**	**47.2**
6	1.978/ 2.03	0.803/ 0.568	1.007	0.437	91.9	36.8	75.7	30.1
9	1.193/ 1.502	0.521/ 1.502	0.474	0.219	98.3	**41.8**	57.1	25.2
12	0.438/ 1.246	0.253/ 1.246	0.169	0.066	**98.7**	34.2	29.6	12.7
24	0.057/ 0.234	0.028/ 0.234	0.017	0.019	18.4	8.4	1.4	0.9
48	0.021 /0.058	0.019/ 0.058	0.004	n.d	2.2	1.5	n.d.	n.d
72	0.013/ 0.031	n.d./ 0.031	0.005	n.d	1.0	1.1	0.3	n.d
96	n.d./ 0.013	n.d/ 0.017	0.003	n.d	0.7	n.d	n.d.	n.d
120	0.005/ 0.021	n.d/ 0.009	n.d.	n.d	0.5	n.d	n.d.	n.d
144	n.d./ 0.006	n.d/ 0.007	n.d.	n.d	n.d.	n.d	n.d.	n.d
168	n.d./ 0.011	n.d/ 0.005	n.d.	n.d	0.3	n.d	n.d	n.d

Table B.5.1.3-2 : Blood and plasma kinetic parameters of pyraflufen-ethyl (pyrazole-5- ^{14}C) after single or repeated oral low or high dose administration (Motoba, 1996a and 1996c)

	1 x 5 mg/kg bw		**15 x 5 mg/kg bw**		**500 mg/kg bw**	
Tissues	plasma	blood	plasma	blood	plasma	blood
Cmax (µg eq./g):						
_	2.8±2.8	1.2±1.3	0.903±0.23	2.68±0.7	100.2±22	44.7±10.7
_	2.6±2.3	1.27±1.1	-	-	107.5±19	48.5±8.2
Tmax (h):						
_	4.8±1.6	5.4±2.5	2.6±2.2	3.8±0.9	7.8±1.6	7.8±1.6
_	3.0±0	3.0±0.0	-	-	4.2±2.7	4.2±2.7
T1/2 (h):						
_	3.5±0.2	4.4±1.6	5.5±1.3	6.1±1.1	7.0±0.4	8.0±3.3
_	3.0±0.5	3.8±1	-	-	3.0±0.3	3.4±0.6
AUC (µg.eq.h/g):						

_	32.3±21.4	14.7±11.7	13.83±1.7	50.5±5.5	2737.6±840	1144.1±403
_	18.3±6	8.7±2.7	-	-	1400.9±915	639.4±381

Distribution:

Regardless to the sex and dose administered, relatively high radioactivity concentrations were observed in excretory organs (intestinal tract, liver, kidney, urinary bladder and gastrointestinal content) at 3, 6 or 9 h postdose. Except stomach, intestinal tract, liver, kidney, and urinary bladder, there were no tissues or organs exhibiting higher radioactivity concentrations than for plasma, which suggested that pyraflufen-ethyl (pyrazole-5- ^{14}C) and its metabolite translocated hardly to tissues and organs. At 96 h post-dose, in all groups, there were no tissues, or organs exhibiting specifically retained radioactivity (Table B.5.1.3 -3 and Table B.5.1.3-4).
Increasing the dose by a factor of 100, decreased the % radioactivity of the administered dose in the reported organs (Table B.5.1.3 -3).

Table B.5.1.3 -3 : Distribution of radioactivity in some tissues after oral administration of pyraflufen-ethyl labelled on Pyrazole-5-^{14}C (Motoba, 1996a).

	Amounts of radioactivity (% of dose) 5 mg or 500 mg/kg bw (5mg/500 mg)					
Tissues	_			_		
sample collection time	**6 or 9h***	**24 h**	**96 h**	**3 or 6 h***	**24 h**	**96 h**
Eyeballs	0.51/ <0.001	<0.001/ <0.001	<0.001/ <0.001	0.002/ <0.001	<0.001/ <0.001	<0.001/ <0.001
Stomach	0.07/ 0.010	0.011/ 0.002	<0.001/ <0.001	**1.13**/ 0.15	0.008/ 0.006	<0.001/ <0.001
liver	**4.21**/ 0.81	0.16/ 0.056	0.007/ 0.004	**5.32**/ 0.570	0.029/ 0.024	0.004/ 0.003
kidney	0.17/ 0.066	0.01/ 0.005	<0.001/ <0.001	**1.36**/ 0.106	0.006/ 0.004	0.001/ <0.001
Small intestine	0.79/ 0.055	0.04/ 0.007	0.002/ <0.001	**5.2**/ 0.52	0.017/ 0.008	0.002/ <0.001
large intestine	**3.2**/ 0.62	0.030/ 0.02	0.001/ <0.001	0.54/ 0.35	0.013/ 0.02	0.002/ <0.001
gastrointestinal contents	**43.7**/ **31**	**1.34**/ 0.74	0.012/ 0.005	**36.8**/ **34.5**	0.58/ **1.75**	0.013/ 0.004
Levels were lower than 0.1% in other tissues. *** after 500 mg/kg, samples were taken at 9 h for males and 3 h after 5 mg/kg bw for females**						

Table B.5.1.3 -4: Distribution of radioactivity in some tissues after repeated oral administration of pyraflufen-ethyl labelled on Pyrazole-5-^{14}C (Motoba, 1996c).

	Amounts of radioactivity (% of dose)		
Tissues	_		
sample collection time	**6 h**	**24 h**	**96 h**
Eyeballs	<0.001	<0.001	nd
Stomach	0.403	0.016	<0.001
liver	4.53	0.235	0.008

kidney	0.203	0.013	0.001
Small intestine	0.640	0.033	0.001
large intestine	1.997	0.085	<0.001
gastrointestinal contents	70.23	1.26	0.008

nd : not detectable

Metabolism:

The test substance is mainly metabolized through ester hydrolysis (giving metabolite E-1 which give rise to E-9 after N-dealkylation), N-demethylation of the pyrazole ring (giving metabolite E-8 further transformed into E-9) which may undergo hydrolysis of ether to phenol and further methylation to methoxy moiety (see figure B.5.1).

Regardless to the sex and dose administered, the main metabolites in urine, feces, bile and plasma were E-9 and E-1. In urine and feces, these metabolites were more important after low than after high dose administration and suggest that ester hydrolysis is a saturable process.
Important level of unchanged compound were detected in feces reaching 78% of the fecal radioactivity after high level. Conjugated metabolites were not identified.
E2, E3, E8, E10 and E11 were detected at trace levels (Table B.5.1.3 -5 and Table B.5.1.3 -7).
Amounts of unknown metabolites and unextractable radioactivity in urine or feces were quite small reaching respectively a maximum of 1 or 3% of dosed radioactivity.
In plasma, after 3 or 6 h oral administration of a low dose of puyraflufen-ethyl, unchanged compound was detected at traces levels (3h) or absent (6 h). Main plasma metabolites were E-9 (representing 75% of the total plasmatic radioactivity) and E-1 reaching 14.4 to 8.6% with time (Table B.5.1.3 -6).

Table B.5.1.3 -5: Quantitation of rat urinary and faecal radioactivity in % of dose of pyraflufen-ethyl labelled on the pyrazole-5-^{14}C (pyr) or on phenyl-U- ^{14}C(-U-): main metabolites excreted within 48 h postdose (Motoba, 1996a and b).

Dose	**Urine (% of dosed radioactivity)**							**Faeces (%of dosed radioactivity)**						
mg/kg bw	**1 x 5**				**15 x 5**	**500**		**1 x 5**				**15 x 5**	**500**	
Sex	_		_		_	_	_	_		_		_	_	_
-^{14}C	pyr	-U	pyr	-U	pyr	pyr	pyr	pyr	-U-	pyr	-U-	pyr	pyr	pyr
Pyraflufen-ethyl	0.09	0.02	0.7	Tr	n.d	0.06	0.1	**17.9**	**19.95**	**14.4**	**27.41**	**4.35**	**78.2**	**78.7**
E1	**3.7**	**1.79**	**9.6**	**6.61**	**2.49**	**0.23**	**1.37**	**28.0**	**34.84**	**38.0**	**36.43**	**43.5**	**5.08**	**6.10**
E2	n.d	nd	tr	nd	tr	tr	0.03	0.24	1.47	0.06	tr	0.62	0.02	0.09
E3	n.d.	nd	n.d	0.07	n.d	n.d.	n.d	n.d.	nd	n.d	nd	nd	n.d	n.d
E8	n.d	nd	n.d	nd	n.d	n.d	n.d	n.d	nd	n.d	nd	nd	n.d	n.d
E9	**23.99**	**14.7**	**22.1**	**13.9**	**23.52**	**3.99**	**4.37**	**17.88**	**18.76**	**12.1**	**12.66**	**15.57**	**4.94**	**1.86**
E10	tr	nd	tr	nd	tr	0.01	tr	0.05	tr	0.12	0.05	tr	0.04	0.03
E11	tr	nd	tr	nd	0.41	n.d.	nd	nd	nd	nd	nd	nd	n.d	nd
other	0.72	0.01	0.31	0.12	0.58	0.48	0.44	0.75	0.28	2.18	tr	0.11	0.67	0.68
unextractable	0.11	0.02	0.15	0.03	0.08	0.03	0.02	2.74	4.2	3.13	4.67	0.36	2.2	1.93
sum	**29**	**16.55**	**33**	**21**	**26.5**	**4.8**	**6.4**	**67.6**	**79**	**70**	**81**	**64.3**	**91.1**	**84**

Tr: traces ; nd: not detected

Table B.5.1.3 -6: Identification of rat plasma radioactivity in % of dose of pyraflufen-ethyl labelled on the pyrazole-5-^{14}C after oral single or repeated administration of 5 mg/kg bw (Motoba,1996a, and 1996c).

Metabolites	**Plasma (% of total plasmatic radioactivity)**		
Dose	**1 x5 mg/kg bw**	**1x5 mg/kg bw**	**15 x 5 mg/kg bw**
	–	–	–
Time of sacrifice	**6h**	**3 h**	**6h**
Pyraflufen-ethyl	nd	0.11	nd
E-1	8.61	14.4	3.32
E-2	0.12	0.29	nd
E-3	0.68	nd	nd
E-8	nd	nd	nd
E-9	75.7	74.74	86.99
E-10	1.29	0.09	nd
E-11	nd	nd	nd
Others	6.52	6.22	13.06
Unextractable	7.09	4.17	7
Total	100	100	100

nd = not detected

Table B.5.1.3 -7 : bile duct cannulated rats : metabolites excretion after single low dose of pyraflufen-ethyl labelled on the Pyrazole-5-^{14}C (Motoba, 1996d).

	Amounts of metabolites (% dosed radioactivity)			
labeling	**Pyrazole-5-^{14}C**			
tissues	**urine**	**feces**	**bile**	**gastrointestinal content**
	–	–	–	–
Pyraflufen-ethyl	nd	1.67	nd	1.19
E-1	**0.13**	**14.03**	**3.39**	**11.19**
E-2	tr	0.02	tr	tr
E-3	nd	nd	nd	nd
E-8	nd	nd	nd	nd
E-9	**19.36**	**0.51**	**27.13**	**0.4**
E-10	tr	tr	tr	tr
E-11	nd	nd	nd	nd

Unknown -1	nd	nd	1.39	nd
Others	0.13	0.03	3.86	0.05
Unextractable	0.03	1.64	0.33	1.02
Total	19.65	17.9	36.11	13.86

Tr = traces ; nd = not detected

Excretion:

In both male and female rats, irrespective of label position, more than 95% of administered dose was excreted within 24 h post-dose.

Urinary excretion represented 28.7-33.2% of the low dose administration and was maximal within ther firts 24 h post-dose. Increasing the dose up to 500 mg/kg bw decreased urinary excretion to 2.5-4.9% and was accompanied by a simultaneous increased fecal excretion of unchanged compound (table B.5.1.3-5). This suggests that bioavailability of pyraflufen-ethyl decreases with increasing the dose (Table B.5.1.3 -8).

Repetitive dosing of pyraflufen-ethyl had no significant effect on rate of excretion and distribution between urine and feces. Radioactivity mainly excreted into feces reaching 62-90% of a low dose (Table B.5.1.3 -8).

Radioactivity into expired air until 24 h postdose was below detection limit or smaller than 0.05% of dosed radioactivity (Table B.5.1.3 -8).

Pyraflufen-ethyl is readily excreted into bile : until 6 h postdose, 6.4% was excreted into bile. Cumulative radioactivity excretion until 48 h postdose reached 36.09% of the dose (Table B.5.1.3 -9).

Table B.5.1.3 -8 : Recovery of radioactivity in urine, expired air and faeces as a function of time after single or repeated low dose or high oral dose of pyraflufen-ethyl (Motoba, 1996a ; Motoba, 1996c).

	Recovery (% dose)						
labeling	**Pyrazole-5-^{14}C**					**phenyl- U-^{14}C**	
dose	**5 mg/kg bw**		**500 mg/kg bw**		**15 x 5 mg/kg bw**	**5 mg/kg bw**	
	_	_	_	_	_	_	_
urine							
0-24	28.04	32.54	3.66	2.38	25.83	16.09	20.67
24-48	0.57	0.42	0.67	0.22	0.69	0.46	0.15
48-72	0.12	0.16	0.41	0.18	0.11	0.18	0.06
72-96	0.06	0.08	0.23	0.06	0.04	0.12	0.02
Total	**28.72**	**33.21**	**4.97**	**2.56**	**26.66**	**16.85**	**20.9**
feces							
0-24	66.77	69.68	90	88.64	61.64	78.86	78.88
24-48	0.86	0.38	1.13	0.72	2.68	0.62	2.34
48-72	0.06	0.06	0.11	1.14	0.08	0.11	0.15
72-96	0.04	0.04	0.03	0.03	0.03	0.08	0.04
Total	**67.72**	**70.16**	**91.27**	**90.53**	**64.44**	**79.67**	**81.41**
cage wash	n.d.	n.d.	1.62	0.28	0.14	0.6	0.19
exp.air	0.03	0.04	-	-	-	nd	nd

total	**96.55**	**103.4**	**97.86**	**97.51**	**91.24**	**97.12**	**102.5**

nd : not detected

Table B.5.1.3 -9 : Excretion of radioactivity in bile of male rats as a function of time after single low dose of pyraflufen-ethyl labelled on the Pyrazole-5-^{14}C (Motoba, 1996d).

Time(h)	Cumulative excretion (% dose)
Biliary excretion:	
0-6	6.4 ± 2.59
6-12	13.48 ± 5.87
12-24	24.13 ± 11.22
24-48	36.09 ± 12.53
Faecal excretion	
0-24	0.9 ± 1.12
24-48	17.9 ± 14.24
Gastrointestinal content :24-48h	
	13.86 ± 9.86
Urinary excretion:	
0-24	15.09 ± 7.64
24-48	19.66 ± 9.75
Total	90.36 ± 4.33

Metabolism of pyraflufen-ethyl in livestock:

No data. Not necessary.

Metabolism of pyraflufen-ethyl in plants:

The metabolism of ^{14}C-phenyl and ^{14}C-pyrazole-Pyraflufen-ethyl has been investigated in spring wheat plants : in the whole wheat plant, metabolism involves initial hydrolysis of the ethyl ester and de-methylation of the pyrazole ring. Further metabolism of the phenoxyacetate group, producing phenol and methoxy groups, was observed in mature straw. In addition to these degradates, up to seven unidentified metabolites were also detected. From these results, it appears that pyraflufen-ethyl is similarly metabolized in plants as in rats.

General conclusion:

After oral administration, pyraflufen-ethyl showed a rapid, dose-dependent and saturable absorption from the gastro-

intestinal tract.
An absorption rate of 56% was extrapolated from urinary and biliary excretion (36.09% in bile + 19.66% in urine) for low dose. Increasing the dose, increased fecal excretion (± 90%) and reduced simultaneously urinary excretion to 3.6%. Absorption after oral high dose represents ± 20% of the dose.
Pyraflufen-ethyl has a limited distribution in tissues and organs, and 96 h after dosing, there were no tissues or organs exhibiting specifically retained radioactivity. At 3, 6 or 9 h postdose, the highest radioactivity was associated with the gastro-intestinal tract and the organs of metabolism and elimination, i.e. liver and kidney.
There was no evidence of accumulation.
Metabolism in rats as well as in plants, involves ester hydrolysis, O-dealkylation of the ether, N-demethylation on the pyrazole ring and further transformations of the phenoxyacetate into more polar metabolites. These metabolic pathways produce essentially 2 metabolites identified as E-1 and E-9 and other metabolites present at very low levels.
Excretion after oral administration was mainly fecal, representing ± 66% of a low dose, reaching 90% after high dose from which ± 78% was unchanged pyraflufen-ethyl. Urinary excretion represented 29-33% of the low dose and decreased to 2.5-5% after high dose. There was no evidence of accumulation.
- Absorption, distribution, metabolism, and excretion of pyraflufen-ethyl (pyrazole-5-^{14}C) following a single oral administration to male and female rats (Motoba, 1996a).
Guidelines :
Experimental protocol in compliance with test method B, directive 87/302/EEC.
GLP :
Yes (no attest of competent autority).
The study is accepted.
Material and methods:
After 16 to 18 h starvation, 5 rats (Sprague-Dawley)/group, received by gavage, a single dose of pyraflufen-ethyl labelled on the pyrazole-5- ^{14}C (b.n°H-251-30 ; specific activity : 60.2 mCi/mol = 2.23 GBq; >98%purity) and non-labelled (b. n° 5AM0027P; purity 99.4%) solubilized in 0.5% CMC-Na containing 0.1% tween 80 to make final Pyraflufen-ethyl concentration of 500kBq/ml ; 5 or 500 mg/kg bw were administered.
Blood was collected at 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144 and 168 h.
For the distribution study animals were sacrificed and blood, organ and tissue samples were collected at 3, 6, 9 and 24 h post dose.
For excretion studies rats were transferred to metabolic cages and urine and feces were collected for 96 h.
Metabolite analysis in urine, plasma or faeces:
An aliquot of urine or plasma was precipitated by addition of acetonitrile; after centrifugation, obtained precipitate was extracted again with acetonitrile. Radioactivity of precipitate was recognized as unextractable. Supernatant was combined and evaporated under N_2 and reconstituted with acetone. Radioanalysis was performed on acetone extract ; an aliquot of acetone solution was analysed with HPLC equipped with radioisotope monitor. Some of the reconstituted acetone solution was applied to 2-dimensional thin layer chromatography.
Faeces were extracted with acetonitrile and centrifugated. The same scheme as for urine was followed.

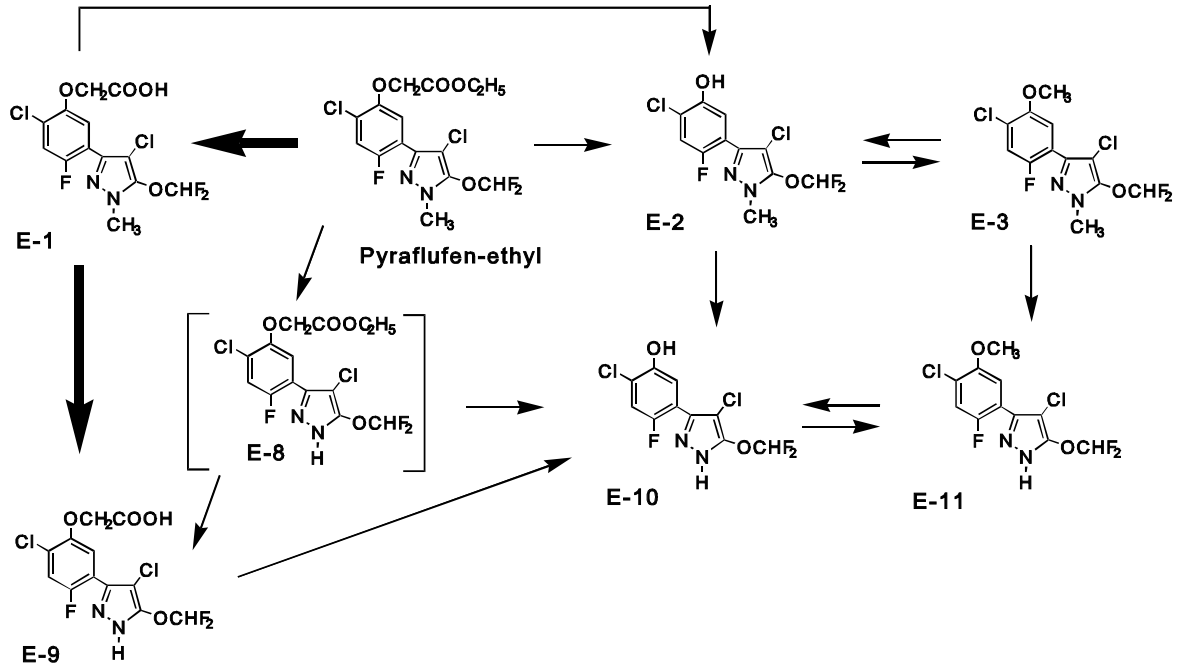
-Absorption, distribution, metabolism, and excretion of pyraflufen-ethyl (pyrazole-5-^{14}C) following 14 + 1 repetitive oral administration to male rats (Motoba, 1996c).
Guidelines :
Experimental protocol not fully in compliance with test method B, directive 87/302/EEC.
Deviation from official protocol: in the excretion studies, radioactivity was not measured until about 95% of the dose has been excreted or for seven days.
GLP :
Yes (no attest of competent autority).
The study is accepted.
Material and methods:
After starvation, 5 male rats (Sprague-Dawley)/group, received by gavage, for 14 consecutive days, 5 mg/kg bw pyraflufen-ethyl (b.n°5AM0027P; purity 99.4%). At the 15 th day, rats received a single administration of pyraflufen-ethyl labelled on the pyrazole-5- ^{14}C (b.n°H-251-30 ; specific activity : 60.2 mCi/mol = 2.23GBqmmol ; 99.9 %purity). The compound was solubilized in 0.5% CMC-Na containing 0.1% tween 80 to make final Pyraflufen-ethyl concentration of 500kBq/ml.
Blood was collected at 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144 and 168 h.
For the distribution study animals were sacrificed and blood, organ and tissue samples were collected at 6 and 24 h post dose.
For excretion studies rats were transferred to metabolic cages and urine and feces were collected for 96 h.
Metabolite analysis in urine, plasma or faeces:
As previously described in Motoba, 1996a.

-Metabolism and excretion of pyraflufen-ethyl (pyrazole-5-^{14}C) following a single oral administration to male rats

(Motoba, 1996d).
Guidelines :
Experimental protocol in compliance with test method B, directive 87/302/EEC.
GLP :
yes (no attest of competent autority).
The study is accepted.
Material and methods:
20 male rats (Sprague-Dawley) were bile cannulated under anesthesia after 16 to 18 h starvation. Rats received received by gavage, 5 mg/kg bw pyraflufen-ethyl labelled on the pyrazole-5- ^{14}C (b.n°H-251-30 ; specific activity : 60.2 mCi/mol = 2.23GBq/mmol ; >98 %purity). Non labelled compound (b.n°5AM0027P; purity 99.4%).The compound was solubilized in 0.5% CMC-Na containing 0.1% tween 80 to make final Pyraflufen-ethyl concentration of 500kBq/ml.
Bile was collected at was collected at0-6, 6-12, 12-24, 24-36 and 36-48 h postdose. Urine were collected every 24 h.

-Metabolism and excretion of pyraflufen-ethyl (phenyl-U-^{14}C) following a single oral administration to male and female rats (Motoba, 1996b).
Guidelines :
Experimental protocol in compliance with test method B, directive 87/302/EEC.
GLP : yes (no attest of competent autority).
The study is accepted.
Material and methods:
5 rats (Sprague-Dawley)/sex were administered orally (after 16 to 18 h starvation), 5 mg/kg bw pyraflufen-ethyl labelled on the phenyl-U- ^{14}C (b.n°H-251-48 ; specific activity : 55.7 mCi/mol = 2.06GBq/mmol ; 99.32 %purity). Non labelled compound (b.n°5AM0027P; purity 99.4%).The compound was solubilized in 0.5% CMC-Na containing 0.1% tween 80 to make final Pyraflufen-ethyl concentration of 500kBq/ml.
Urine and fecal samples were collected at every 24 h until 96 h postdose. CO_2 and organic volatile in expired air were trapped with monoethanolamine ; the trap was changed every 24 h.

Figure B.5.1 : Metabolic pathway of pyraflufen-ethyl in rats

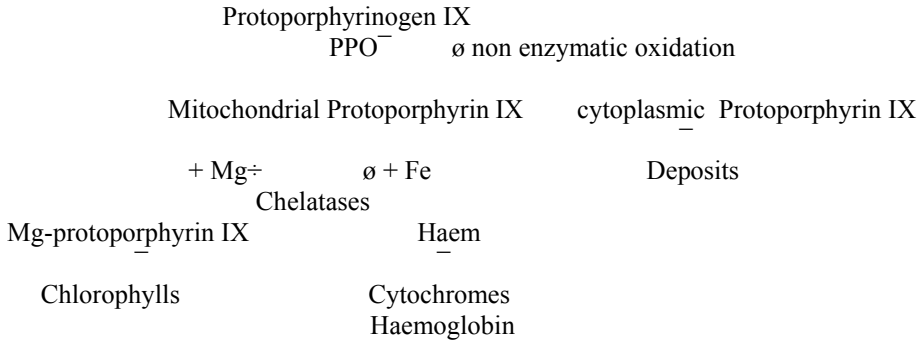


E-1 = 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazole-3yl)-4-fluorophenoxyacetic acid)
E-2 = 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazole-3yl)-4-fluorophenol
E-3 = 4-chloro-3-(4-chloro-2-fluoro-5-methoxyphenyl)-5-difluoromethoxy-1-methylpyrazole
E-8 = ethyl 2-chloro-5-(4-chloro-5-difluoromethoxylpyrazole-3yl)-4-fluorophenoxyacetate
E-9 = 2-chloro-5-(4-chloro-5-difluoromethoxylpyrazole-3yl)-4-fluorophenoxyacetic acid
E-10= 2-chloro-5-(4-chloro-5-difluoromethoxylpyrazole-3yl)-4-fluorophenol
E-11 =4-chloro-3-(4-chloro-2-fluoro-5-methoxyphenyl)-5-difluoromethoxylpyrazole

B.5.2 Acute toxicity including irritancy and skin sensitization (Annex IIA 5.2)

Introduction : data from the open literature:

Mechanism of action:
Pyraflufen-ethyl acts as an inhibitor of protoporphyrinogen IX oxidase (PPO).
PPO is one of the key enzymes in porphyrin biosynthesis, common to both plants and animals, as part of chlorophyll and heme synthesis.
PPO (located in mitochondria) catalyzes the six-electron oxidation of protoporphyrinogen IX to protoporphyrin IX.
In case of enzyme inhibition, Protoporphyrinogen IX accumulates and diffuse into other cell compartments where it is oxidized into protoporphyrin either non specifically by membrane peroxidases or, non enzymatically. Cytoplasmic protoporphyrin PPIX is not a substrate for mitochondrial ferrochelatase and accumulates. Protoporphyrin forms in liver tissue pigment cristallin deposits, visible by polarisation and electron microscopy (Bloomer 1997). Protoporphyrin is photosensitive and generates reactive oxygen species in light (Rio et al., 1997).



Human diseases:
Porphyrias are disorders of porphyrin or porphyrin-precursor metabolism that result from inherited or aquired aberrations in the control of the porphyrin-heme biosynthetic pathway. Deficiency in activity of PPO results in the human genetic disease *variegate porphyria*. This metabolic disease (called porphyria) is characterized by accumulation of porphyrins in hepatocytes (Krijt et al., 1993) and acute excretion of copro- and protoporphyrins (Rio et al., 1997). The demonstration of increased fecal excretion of porphyrin is frequently used, but levels may be normal (Logan et al., 1991).
Clinical symptoms or porphyria include severe abdominal pain, vomiting, constipation, hypertension, tachycardia and bladder dysfunction. These symptoms have been ascribed to autonomic neuropathy. Other symptoms are motor weakness and sensory involvement, which correlate with peripheral axonal neuropathy, and mental symptoms occurring without clear morphological findings in the cerebrum (Meyer et al., 1998). Dermatological changes, which present clinically as cutaneous photosensitivity are caused by the photosensitizing properties of circulating porphyrins (Moore, 1993)
All cytochrome P450 enzymes contain heme as a prosthetic group and their expression could be modified by porphyrogenic agents (Salonpaa et al., 1997).
Inhibition of protoporphyrinogen oxidase may cause embryolethality, teratogenicity, and growth retardation due to haematological dysfunction caused by inhibition of heme biosynthesis (Kawamura et al., 1996)
An increased incidence of hepatocellular carcinoma has been reported in subjects (particularly in women) with *porphyria cutanea tarda* and is usually associated with an underlying liver disease (Andersson et al., 1996 ; Andant et al., 1998).

B.5.2.1 Acute oral toxicity (Annex IIA 5.2.1)

- Rat, limit test at 5000 mg/kg bw (Amanuma, 1995a)

Findings :
Mortality : there was no death until 14 days after dosing.
Body weight : no changes.
Clinical observations : no compound related effect.
Ne cropsy : no abnormality.

Conclusions :
LD_{50} male >5000 mg/kg bw
LD_{50} female >5000 mg/kg bw
LD_{50} combined >5000 mg/kg bw.

Guidelines :
Experimental protocol in compliance with method B.1 Annex V of Directive 84/449/EEC.
GLP :
Yes (no attest of competent autority)
Material and Methods :
5 Sprague-Dawley rats/sex/group received a single intragastric intubation of Pyraflufen-ethyl technical (B.n°.4AM0021D ; 97.5%) incorn oil at 5000 mg/kg bw.
The study is accepted.

- Mice, 2000 and 5000 mg/kg bw (Amanuma, 1995b)

Findings :
Mortality : there were no deaths in either sex for 14 days after dosing.
Body weight : not affected by treatment.
Clinical observations : dirty and coarse fur was observed in all treated animals ; decrease in locomotor activity in 1 male at top dose.
Necropsy : 1 male at 5000 mg/kg bw showed atrophy of the thymus.

Conclusions :
Pyraflufen-ethyl is slightly more toxic for males than for females.
LD_{50} male >5000 mg/kg bw
LD_{50} female>5000 mg/kg bw
LD_{50} combined >5000 mg/kg bw.

Guidelines :
Experimental protocol in compliance with method B.1 Annex V of Directive 84/449/EEC.
GLP :
Yes (no attest of competent autority)
Material and Methods :
5 slc : ICR mice/sex/group received a single intragastric intubation of Pyraflufen-ethyl technical (B.n°.4AM0021D ; 97.5%) in corn oil at 2000 and 5000 mg/kg bw.
The study is accepted.

B.5.2.2 Acute percutaneous toxicity (Annex IIA 5.2.2)

- Rat, 2000 mg/kg bw, semi-occluded dressing for 24 h. (Amanuma, 1995c)

Findings :
Clinical signs : Tearing was observed in 1 female only at 5 hours after application.
Body weight of male rats showed a tendency of decrease on 7th day after application, and showed significant low value(¯ 4%) on 14th day after application.
Necropsy findings: the formation of white foci, being the size of adzuki beans, on the head of the right epididymis

was observed in 1 male and a round cyst, being the size of 5 mm in diameter, on the right ovary was observed in 1 female. It was not clear wether these effects are the result from treatment.

Conclusions :
LD_{50} male > 2000 mg/kg bw
LD_{50} female > 2000 mg/kg bw
LD_{50} combined > 2000 mg/kg bw

Guidelines:
Experimental protocol in compliance with method B.3 Annex V of Directive 92/69/EEC.
Comment : females were 12 week old and males 5 week old.
GLP :
Yes (no attest of competent autority)
Material and Methods :
5 Slc : SD rats/sex were dermally exposed to Pyraflufen-ethyl technical (B.n°4 AM0021D; 97.6.%) as a paste with distilled water at 2000 mg/kg bw under semi-occluded dressing for 24 h. The study is accepted.

B.5.2.3 Acute inhalation toxicity (Annex IIA 5.2.3)

-Rat, inhalation of dust by nose only exposure, 5.03 mg/l, 4 hour, (Cracknell, 1995)

Findings :
Clinical signs: non-specific response to the inhalation of a particulate aerosol such as exaggerated respiratory movements and reduced respiratory rate were noted. 30 minutes after the exposure was completed, wet fur was observed for all of the animals that had been exposed to pyraflufen-ethyl.
Body weight: no effect was noted.
Necropsy: organ weights were not affected.
Conclusions :
4-hour LC_{50} > 5.03 mg/l
$\left(\frac{5.03 \times 7\ l \times 1000\ mg/kg \times 4\ hours}{333} > 423\ mg/kg\ bw\right)$

Guidelines :
Protocol in compliance with method B.2, Annex V of directive 92/69/EEC.
GLP :
Yes (no attest of competent authority)
Material and Methods :
5 CD strain rats/sex were exposed nose only to 5.03 mg/l Pyraflufen-ethyl technical (B.n°. 4AM0024D; 97.0%). A dust feed mechanism was used to produce the test atmosphere.The mechanism was designed to produce and maintain an atmosphere containing dust by suspending material scraped from the surface of a compressed powder in a stream of dry air.
The MMAD was calculated to be 11.48µm; 29.3% were considered to be of inhalable aerodynamic particle size.
The study is accepted.

B.5.2.4 Skin irritation (Annex IIA 5.2.4)

- Rabbit, semi- occlusive dressing, 500 mg/kg bw, 4 hours (Komatsu, 1995a)

Findings
There were no irritation at the intact or at the abraded skin of all animals treated with both the test substance and distilled water during the observation periods. There was no changes in animals body weights.
Evaluation of the data according to the EU methodology, gave the following results:

< Score erythema > 24+48+72 h = 0
< Score oedema > 24+48+72 h = 0

Conclusions :
Pyraflufen-ethyl is not a skin irritant.

Guidelines :
Protocol in compliance with method B.6 of directive 92/69/EEC or OECD guideline 406(1981)
GLP :
Yes (no attest of competent autority)
Material and Methods :
Shaved abraded and non-abraded skin of 6 male pathogen free Japanese white rabbits was exposed to 500 mg of Pyraflufen-ethyl wetted with 1 ml distilled water (B.n°.4AM0021D ; 97.6%) under semi-occluded dressing for 4 hours.
The study is accepted.

B.5.2.5 Eye Irritation (Annex IIA 5.2.5)

- Rabbit eyes, 100 mg (Komatsu, 1995b)

Findings :
One hour after instillation, slight congestion of the iris was observed in 3/6 animals in the unrinsed group. This change remained untill 24 h after instillation in 1 animal out of the 3, but disappeared up to 48 h after instillation. Slight congestion in the conjunctivae was observed one hour after instillation, in 5 animals out of the 6 in the unrinsed group.This change remained untill 24 h after instillation in 1 animal out of the 5, but disappeared up to 48 h after instillation.
Evaluation of the data, according to the EU methodology, gave the following results for the unrinsed eyes of 6 rabbits :

< Score cornea opacity > 24+48+72h = 0
< Score iris > 24+48+72 h =0.0555
< Score redness > 24+48+/72h =0.0555
< Score chemosis > 24+48+72 h=0

Conclusions :
Pyraflufen-ethyl is not irritating to eyes.

Guidelines :
Protocol in compliance with method B.5 of Directive 92/69/EEC.
GLP :
Yes (no attest of competent autority)
Material and Methods :
100 mg pyraflufen-ethyl (B.n°. 4AM0021D ; 97.6%) was placed into the left eye of each of 9 male Japanese white rabbits. The eye of three animals were rinsed using warmed water.
The study is accepted.

B.5.2.6 Skin sensitization (Annex IIA 5.2.6)

- Guinea pig, Maximization test (Rees, 1995)

Findings :
Induction performed with 10% in propylene glycol caused moderate erythema, with 10% in propylene glycol and in adjuvant caused moderate erythema and pallor.
Epidermal application with 30% w/v pyraflufen-ethyl in propylene glycol under occlusive dressing, gave rise to isolated cases of exfoliation.
Challenge application of 30%, on day 21, gave rise to no dermal reaction in any animal; barely perceptible erythema in 1 test animal was observed after challenge application of 5% pyraflufen-ethyl in propylene glycol.
Conclusions :
Pyraflufen-ethyl is not a sensitizer.

Guidelines :
Protocol in compliance with method B.6, Annex V, Directive 92/69/EEC (Maximisation test)
GLP :
Yes (no attest of competent autority)
Material and Methods :
10 Albino guinea pigs (Dunkin-Hartley)/sex were exposed to pyraflufen-ethyl (B.n°. 4AM0024D; 97.0%)
In the control group 5 animals/sex were used.
Pre-treatment formulation trials indicated that 10% w/v pyraflufen-ethyl in propylene glycol was the maximum injectable concentration. For topical administration, the maximum practicable concentration was a 50% solution.
Induction was performed with 10% in propylene glycol (w/v), and with 10% FC. Epidermal application on day 8 was performed with 30% w/v pyraflufen-ethyl in propylene glycol under occlusive dressing. For challenge, on day 21, the test group was treated with 30% or 5% in propylene glycol, the control group with propylene glycol only.
The study is accepted.

B.5.2.7 Summary of acute toxicity including irritancy and skin sensitization (Annex IIA 5.2)

In rats and mice, acute oral toxicity was low and no mortality occurred after oral administration of 5000 mg/kg bw pyraflufen-ethyl. Moreover, no particular symptoms were recorded in rats. Male mice appeared more sensitive and some symptoms suggestive of an autonomic nervous sytem toxicity were observed.
From supplementary studies performed in male mice (point B.5.8), it appeared that a single oral dose of 5000 mg/kg bw was without significant effect on liver weight, on cytochrome P450 level and on the enzyme activities which are cytochrome P450 dependent. At a higher dose, 10000 mg/kg bw, inhibition of cytochrome P-450 dependent activities occurred, as a result of interference of pyraflufen-ethyl with the heme synthesis.

Dermal toxicity was low but at the dose of 2000 mg/kg bw, male rats showed a significant reduced body weight at the 14th day.
Pyraflufen-ethyl is not an eye and skin irritant, and is not a sensitizer.

Table B.5.2.7-1 : Summary of acute toxicity of pyraflufen-ethyl

Type of test; test species	**Test substance purity**	**Results**	**Classification**	**References**
Acute oral, rat	97.5% batch n°.4 AM0021D	_ _ >5000 mg/kg bw	-	Amanuma, 1995a
Acute oral, mice	97.5% batch n°.4 AM0021D	_ _ >5000 mg/kg bw	-	Amanuma, 1995b
Rat, dermal, semi-occluded	97.6% batch n°.4 AM0021D	_ _ >2000 mg/kg	-	Amanuma, 1995c
Rat, nose only inhalation, 4 hours (dust)	97.0% B.n°. 4AM0024D	_ _ > 5.03 mg/l (423 mg/kg bw)	-	Cracknell, 1995
Rabbit, skin irritation	97.6% B.n°.4AM0021D	_: not irritant	-	Komatsu, 1995a
Rabbit, eye irritation	97.6% B.n°. 4AM0021D	_: not irritant	-	Komatsu, 1995b
Maximisation test	97.0%; B.n°. 4AM0024D	not sensitizer	-	Rees, 1995

B.5.3 Short-term toxicity (Annex IIA 5.3)

B.5.3.1 Oral 28-day toxicity (Annex IIA 5.3.1)

-Dietary administration to rats at 20, 200, 2000 or 20000 ppm, 28 days (Broadmeadow, 1994a)

Findings :
Main findings are described in Table B.5.3.1-1.
The MTD was reached.
Clinical signs and mortality : signs observed prior to death included thin build, underactive behaviour, pallor, partially closed eyelids, respiratory distress and hunched posture. In addition, firm distended abdomen, piloerection and reduced body temperature were also observed in some of these animals. All deaths are clearly related to treatment.
At necropsy, dark liver, swollen spleen, abnormal content or distention of the stomach and gastro-intestinal tract, oedematous thymus and pancreas, fluid in the abdomen, were reported.
In the animals which died or were killed prematurely, diminished glycogen in the liver, lymphoid atrophy in spleen, submucosal oedema in the stomach, oedematous lesion of the pancreas in both males and one female were reported.
Bodyweight: during the first 10 days of treatment low bodyweight gains or bodyweight losses were observed in the top dose animals. Thereafter, the weight gains of males remained slightly lower whilst those of females were similar to those of the controls.
Water consumption: during the first week of treatment, animals of top dose group appeared to be drinking more water than the controls. This effect was not apparent during week 4.
Hematology: treatment related changes were increased reticulocyte numbers, particularly in males, and increased normoblast numbers in males only. Examination of blood smears indicated morphological changes in the erythrocytes comprising anisocytosis and hypochromasia.
Bone marrow analysis indicated a reduction in the myeloid to erythroid ratio in males only at the top dose. Cellularity and composition of the marrow was unaffected.
Organ weight changes and clinical chemistry:
At the top dose, liver weight increase was associated with an increase in AP, plasma transaminases activities, cholesterol and protein concentrations indicating hepatocellular toxicity.
Increase in kidney weight was associated to a minor renal effect, as suggested by a reduced plasma chloride concentrations and an apparent increase in water intake.
The high spleen weights in males treated at 20000 ppm correlated with the observations of extramedullary haematopoiesis in the spleen at microsocopic examination. These changes suggest a compensatory increase in red blood cell production. The slightly high plasma concentration of bilirubin may also be related to these findings and suggests a haemolytic anemia.

Table B.5.3.1-1 : 28 days subacute study in rats.

Endpoints	**Dose (ppm)**									
	0		**20**		**200**		**2000**		**20000**	
	_	_	_	_	_	_	_	_	_	_
Mortality									2	2
Clinical signs									see text	
Body weight									î18%	
Body w. gain									î28%	
Food cons. (%)			105	95	104	94	105	95	101	92
Hematology:										
PCV									î16%	î7%
Hb									î22%	î11%
MCV									î11%	î8%
MCH									î20%	î11%

Endpoints	Dose (ppm)									
	0		20		200		2000		20000	
	_	_	_	_	_	_	_	_	_	_
WBC : Neutro.									ì85%	
WBC: leuco.									ì113%	ì44%
Reticulocytes: %	2.5	2							14.4±11	4±1.2
Myeloid: erythroid ratio									î33%	
Morphological variations in blood smears: nbre affected rats										
anisocytosis					1/10		1/10		8/8	8/8
hypochromasia									8/8	8/8
normoblasts present									6/8	
Clinical chemistry :										
AP							î22%		ì17%	
ALT									ì55%	
AST									ì27%	
urea									ì12%	î29%
bilirubin total									ì50%	ì50%
cholesterol total									ì88%	ì69%
Total plasma protein:									ì5%	
albumin									ì9%	
α1 globulin									ì22%	
Cl^-									î5%	î5%
Relative organ weights :										
liver					ì10%		ì17%		ì50%	ì19%
kidneys									ì13%	
spleen									ì26%	ì29%
Microscopic examination : nbre affected rats										
spleen extramedullary hematopoiesis									4/8	
Statistically significant intergroup difference(ì or î ; $p<0.01$) in haematology, bone marrow myeloid/erythroid ratio and clinical chemistry according to Student's t'test ; organ weights and bw changes according to Barlett's test; pairwise comparisons according to Behrens-Fisher test or Dunnett's test; two-tailed Fisher's Exact test.										

Conclusions :
A decrease in AP as reported at 2000 ppm is mainly consecutive to fasting and results probably from the intestinal isozyme which is an important component of the serum enzyme activity. Therefore, this effect is not to be taken into account for the NOAEL.

The increase in liver weight reported at 200 and 2000 ppm is not accompanied by enzymatic modifications indicatives of liver injury and is therefore, not taken into account.
NOAEL = 2000 ppm = 223.2 mg/kg bw/d

Guidelines :
Experimental protocol not fully in compliance with test method B.7, Annex V, of Directive 92/69/EEC.
Deviation from official protocol : no histological examination of adrenals, testes and heart.
GLP :
Yes (no attest of competent authority)
Material and Methods :
10 CD rats/sex/dose, received in the diet pyraflufen-ethyl (B.n°.3AM0011N; 96.8 %)) at 20, 200, 2000 or 20000 ppm/day, seven times per week, 4 weeks.
Achieved dosage: male : 2.4, 23.2, 230.4, 2619 mg/kg bw/d
females : 2.3, 22.2, 223.2, 2296.4 mg/kg bw/d
The study is accepted.

-Dietary administration to mice at 3000,10000, 30000 ppm, 28 days (Takahashi, 1994)

Findings :
Main findings are described in Table B.5.3.1.2. Because of mortality of all animals at the top dose, this dose was omitted from the table.The MTD was reached.
Clinical signs and mortality : at the top dose, male and females were dead or killed *in extremis* following marked debility within 1 week of treatment. Males and females presented decreased spontaneous motor activity, and soiled or wetted fur in external genital region. Males presented also abnormal respiration, hypothermy, ptosis, lacrimation of eye, distended abdomen.
Bodyweight: female body weights were not affected.
Food efficiency was normal in all groups.
Hematology: microcytic and hypochromic anemia was observed in both sexes treated at 10000 ppm. This effect is probably related to the compound and an increase of platelets observed in the group can be compensatory reaction of the bone marrow against anemia.Slight anemia was also noted in males and females in the 3000 ppm group (significant effects different from control at 5% level).
Clinical chemistry: the increase of serum enzymes and the decrease of glucose and triglycerides suggested liver dysfunction attributable to pyraflufen-ethyl. ALT was elevated, reflecting alteration in cell membrane function and hepatocellular damage. Total bilirubine was increased and is probably related to an hemolytic anemia. The increase in in Ca^{++} and in creatinine (not associated with other effects) in males, and the increased GGT (small fluctuation) in females were unlikely to be toxicologically relevant.
Organ weight changes: were considered to be related to the treatment.
Macroscopic lesions detected in animals found dead or killed *in extremis*: distended stomach with food (6 _;6 _), cecum with hardening of contents (6 _; 6 _) and urinary bladder distended with urine (5 _).
At 10000 ppm, enlargement, accentuated lobular pattern, dark coloured liver, and enlargement of the spleen.
In the 3000 ppm, accentuated lobular pattern and enlargement were noted in liver.

Table B.5.3.1-2 : 28 days subacute study of pyraflufen-ethyl in mice.

Endpoints	**Dose (ppm)**					
	0		**3000**		**10000**	
	_	_	_	_	_	_
Mortality			see text			
Clinical signs			see text			
Body weight			î2-5%		î2-5%	
Food consumption					î32%	
Hematology:						

Endpoints	Dose (ppm)					
	0		3000		10000	
	_	_	_	_	_	_
RBC			î13%		î19%	
PCV or hematocrit			î14%	î6%	î25%	î10%
Hb			î12%	î8%	î25%	î12%
MCV						î7%
MCH					î8%	î10%
Platelet					ì34%	ì31%
Clinical chemistry :						
AP					(ì179%*)	
AST					ì279%	ì75%
ALT					ì1473%	ì566%
glucose					î22%	(î12%*)
creatinine					ì25%	
triglycerides			î29%	î35%	î41%	î49%
bilirubin total					ì110%	ì29%
GGT						ì100%
Ca^{++}					(ì5%*)	
Urinalysis:						
pH					î	î
Relative organ weights :						
liver					ì37%	ì32%
kidneys						(ì10%*)
spleen				(î22%*)	(ì33%*)	
Necropsy findings:liver						
enlargement				1	3	3
dark colour					3	
accentuated lobular pattern			2	3	2	5
Statistically significant intergroup differenceì or î (p<0.01) bw, food cons., hematology, clinical chemistry ; organ weights and bw changes according to Barlett's test; when stat.analysis of variance significant, Dunnett's or Scheffé's test; when heterogenous, evaluation according to Kruskal-Wallis. Urinalysis assessed by Mann-Whitney's U test. Fisher's exact test for clinical signs, mortality, incidence of gross lesions at necropsy. (*ì or î at 5% level of probability)						

Conclusion:
NOAEL< 3000 ppm.

Guidelines :
Experimental protocol not fully in compliance with test method B.7, Annex V, of Directive 92/69/EEC.
Deviation from official protocol : the highest dose induced death and was therefore to high; the lowest dose did not demonstrate a NOAEL.Leucocyte count was not performed. No histological examination on preserved tissues. A new study, reported at point B.5.8 describes histopathological findings.
GLP :
Yes (no attest of competent authority)

Material and Methods :
6 ICR (Crj:CD-1) mice/sex/dose, received in the diet pyraflufen-ethyl (B.n°.3AM0019P; 97.6 %) at 0, 3000, 10000 or 30000 ppm/day, for 4 weeks.
Achieved dosage: male : 441.8, 1414 mg/kg bw/d.
females : 491.9, 1682 mg/kg bw/d.
The study is accepted.

B.5.3.2.1 Oral 90-day toxicity (rat) (Annex IIA 5.3.2)

- Dietary administration of pyraflufen-ethyl to rats for 13 weeks at 200, 1000, 5000, and 15000 ppm, followed by a reversibility period of 8 week (Broadmeadow, 1994b)

Findings:
The main findings are described in Table B.5.3.2.1-1. The MTD was reached.
Mortality and clinical signs were clearly associated to treatment.
At top dose, 3 males died and signs observed prior to death were thin build, underreactive behavior, partially closed eyelids and hunched posture. These signs were associated with piloerection and a swollen abdomen in one animal. In surviving animals irregular or fast breathing and pallor were observed between week 2 and 5. Females were also affected.
Hematology: the reduced erythrocytes characteristics observed at the top dose suggest a mild anemia but the bone-marrow remained normal. Examination of the blood smears indicated marginal morphological changes in the erythrocytes comprising anisocytosis and spherocytosis in some animals receiving 15000 ppm. The high spleen weights did not correlate with any histopathological changes but may related to the haematological changes.
Urinalysis: increased urinary volume and low specific gravities in males receiving the top dose and higher water intake suggest a minor renal effect not associated with histopathological alterations.
Clinical chemistry: AP, ALT and AST were increased in males at the top dose and suggest an effect on the liver. The variations in plasma cholesterol, glucose and plasma proteins were also likely related to such an effect.
Histopathology and electron microscopy: at the top dose, high incidence of periacinar hepatocytic hypertrophy ; lysosomes containing flocculent, electron dense material within hepatocytes and Kupffer cells and increase in amount of smooth endoplasmic reticulum within periacinar hepatocytes, electron lucent vacuoles in hepatocytic mitochondria.
Examination performed during the recovery period, 3 weeks later, indicated a slow hematological recovery which was lower in males than in females*;* there was a partial or complete recovery in clinical chemistry affected parameters.White cell counts (primarily neutrophils) were increased in males ; this observationcould be considered to represent a secondary effect related to the regenerative response to the observed anemia.
Periacinar hepatocytic hypertrophy, observed after 13 week treatment was no more apparent after completion of the reversibility period. (Table B.5.3.2.1-2).

Table B.5.3.2.1-1 : 90 days toxicity study in rats with pyraflufen-ethyl

Endpoints	**Dose (ppm)**									
	0		**200**		**1000**		**5000**		**15000**	
	_	_	_	_	_	_	_	_	_	_
Mortality									3	
Clinical signs									see text	
Body weight gain									î22%	
Food consumption									î week 1,2	

Endpoints	**Dose (ppm)**									
	0		**200**		**1000**		**5000**		**15000**	
	_	_	_	_	_	_	_	_	_	_
Ophthalmology			no changes related to treatment							
Hematology										
hematocrit									î8%	
Hb				î5%					î7%	
RBC				î7%						
MCV									î8%	î8%
MCH									î6%	
Total WBC									ì31%	ì43%
lymphocytes										ì41%
Bone marrow			unaffected by treatment							
Clinical chemistry										
AP									ì96%	
ALT									ì376%	
AST									ì11%	
albumin									î8%	
α1 globulin									î27%	
β-globulin									ì23%	
Glucose				ì19%					î14%	
P										ì19%
Urinalysis:										
volume									ì	ì
pH					î		ì			ì
specific gravity									î	î
Relative organ weight:										
spleen									ì21%	
kidney									ì23%	
Microscopic pathology:										
periacinar hepatocytic hypertrophy							2/10		7/10	
statistically significant intergroup difference(ì or î ; $p<0.01$) in haematology, bone marrow m/e ratio, clinical chemistry was assessed by Student't' test; organ and bw assessed according to Bartlett'stest and Behrens-Fisher test or Dunnett's test. Fisher exact test, two-tailed for pathology.										

Table B.5.3.2.1-2: Haematology after 3 week of the reversibility period

Endpoints	**Dose (ppm)**							
	0		**5000**	**15000**				
Reversibility period				**3 weeks**			**5 weeks**	**7 weeks**
Sex	_	_	_	_	_	_	_	_

Hematocrit			î7%		(î6.5%)	(î2%)
Hb			î7%		î8%	î2%
RBC				ì8%		
MCV			î6%		(î4%)	
Total WBC			ì44%		(ì8%)	
Neutrophils			ì149%		ì48%	
Statistically significant intergroup differenceì or î ; p<0.01 or not significant () was assessed by Student't' test						

Comment :
Because heme synthesis is interrupted, iron is not incorporated in the porphyrin ring during the last step of heme synthesis, and accumulates in the mitochondria.
From the open literature, it appeared that in case of iron overload, iron accumulates selectively in the liver and spleen, especially in the hepatocytes, Kupffer cells and splenic macrophages. Excess iron is stored in the lysosomes of hepatocytes, and when the haemosiderin (degradation product of hemoglobin) increases to toxic levels, signs of organelle alteration followed by cellular death are observed. Iron overload is implicated as a causative factor in liver damage (Omara et al., 1993).

Conclusions :
NOAEL =5000 ppm = 455.5 mg/kg bw/d.

Guidelines :
Experimental protocol in compliance with test method B, annex V, directive 87/302/EEC.
GLP :
Yes (no attest of competent authority).
Material and methods:
10 rats (CD strain)/sex/dose were exposed to (B.n°.3AM0011N; 96.8 %) in diet at 0, 200, 1000, 5000, 15000 ppm for 90 days. 4 rats/sex were added in group 5000 and 15000 ppm and in control for reversibility evaluation after 8 weeks recovery.
Electron microscopical investigation was carried out on liver samples from 3 males and 3 females which ahd received 15000 ppm for 13 weeks and on samples from a similar number of control animals.
Daily dose intake for males: 17.8, 85.6, 455.5, 1489.4 mg/kg bw/d.
For females : 19.4, 95.4, 499.0, 1502.9 mg/kg bw/d.
The study is accepted.

- Mice, diet, 0, 200, 1000 or 5000 ppm, 13 week : satellite group of carcinogenicity study (Kuwahara, 1996)

Findings:
Main findings are described in table B.5.3.2.1-2.
At the top dose, male rats were anemic and liver of males and females showed significantly increased incidences of pale in colour, accentuated lobular pattern, coarse surface, spots and/or masses in the liver, associated with an increased liver weight.

Table B.5.3.2.1-2 : 13 week study of pyraflufen-ethyl in mice.

Endpoints/dose	**0 ppm**		**200 ppm**		**1000 ppm**		**5000 ppm**	
	_	_	_	_	_	_	_	_
Mortality	0	0	0	0	0	0	0	0
Clinical signs			no effect					
Body weight			no effect					
Food consumption					ì			
Food efficiency					î11%			

Endpoints/dose	0 ppm		200 ppm		1000 ppm		5000 ppm	
	_	_	_	_	_	_	_	_
Haematology								
Ht							î13%	
Hb							î13%	
RBC							î9%	
WBC							î48%	
PLT								ì48%
Macroscopic findings :								
liver : pale	0	0	0	0	0	0	10/10*	10/10*
accentuated lobular pattern	1	0	1	0	1	0	7/10*	9/10*
Organ weight								
liver(a)/(r)							ì22% /	ì28%/ì25%
Histopathology: (* and** significantly different from control at 5 and 1% levels of probability, respectively)								
Liver:								
centrilobular hepatocellular swelling	0	0	2	0	9**	0	10**	9**
hepatocellular vacuolation	0	0	2	0	6**	0	10**	9**
ìBrown pigment deposition of Kupffer cells	0	0	0	0	0	0	3	5*
micro-granuloma	5	3	3	7	6	5	10*	9**

Statistical significance was evaluated by Bartlett's test, Dunnett's or Scheffé's multiple comparison test were applied, Kruskal-Wallis non-parametric analysis of variance and Fisher's exact probability test ; a= absolute and r = relative.

Conclusion:
NOAEL = 200 ppm = 20 mg/kg bw/d

Guidelines:
Experimental protocol in compliance with test method B, annex V, directive 87/302/EEC.
GLP :
Yes (no attest of competent authority)
Material and methods:
50 ICR (Crj:CD-1) mice/sex/dose received pyraflufen-ethyl (b.n°.4AM0021D: 97.6%; b.n°. 4 AM0023D, 98%) in the diet at 0, 200, 1000, and 5000 ppm, over a period of 78 weeks. Additional groups of 10 males and 10 females for interim sacrifice after treatment for 13 weeks were allocated at each dose.
Achieved intake :
Male: 0, 21, 109.7, and 546.8 mg/kg bw/d
female :0, 19.6, 98.3, and 523.7 mg/kg bw/d
The study is accepted.

B.5.3.2.2 Oral 90-day toxicity (dog) (Annex IIA 5.3.2)

- Dogs, 3 months, 0, 40, 200, or 1000 mg/kg bw/day in capsules (Broadmeadow, 1996a)

Findings:
Oral administration of pyraflufen-ethyl for 13 weeks at dosages up to 1000 mg/kg bw/d, the maximum recommended by the EC guidelines, did not elicit any toxic responses. The compound was well absorbed as two major metabolites were detected in the plasma, one hour after administration on one day in week 13, though the parent material was not detected.
No mortality was recorded ; clinical signs, body weight, food intake, ophtalmoscopy, peripheral blood and bone marrow, clinical chemistry, organ weights and histopathology were not affected by treatment.
Conclusion:
NOEL= NOAEL = 1000 mg/kg bw/d

Guidelines:
Experimental not protocol in compliance with test method B, Annex V, of directive 87/302/EEC or OECD guideline 409 (1981)
GLP :
Yes (no attest of competent authority)
Material and methods:
4 Beagle dog/dose/sex received pyraflufen-ethyl by capsule (97%; batch n°4AM0024D) at 0, 40, 200, or 1000 mg/kg bw/day for 90 days.
The study is accepted.

B.5.3.2.3 Oral 1 year toxicity (dog) (Annex IIA 5.3.2)

- Dogs, orally, 0, 40, 200, 1000 mg/kg bw/d for 1 year (Broadmeadow,1996b)

Findings:
The MTD was not reached.
Oral administration of pyraflufen-ethyl for 52 weeks at 1000 mg/kg bw/day, did not elicit any toxic response in dogs There were no signs related to treatment and no animals died. Bodyweight and food and water consumption were not affected by treatment. Veterinary and ophthalmoscopic examinations revealed no changes that were attributed to treatment. There were no toxicologically significant changes in blood, bone marrow or urine. Organ weights, macroscopic pathology and microscopic pathology showed no findings related to treatment with pyraflufen-ethyl.

Conclusion :
NOAEL > 1000 mg/kg bw/d.

Guidelines:
Experimental protocol not fully in compliance with test method B, Annex V, directive 87/302/EEC or OECD guideline 409 (1981).
Deviation from official protocol : ornithine decarboxylase was not measured; blood and urinary analysis were not performed at 3 months.
GLP :
Yes (no attest of competent authority)
Material and methods:
6 Beagle dog/dose/sex received by capsule pyraflufen-ethyl (97.3%; batch n°EN31653 ; 5AM0025D, 97.7% used up to week 51 and b.n°.5AM0026D was used to complete the study) at 0,40,200 or 1000 mg/kg bw/d for 52 weeks.
The study is accepted.

B.5.3.3.1 28-day inhalation toxicity (rat) (Annex IIA 5.3.3)

In the acute inhalation toxicity studies of pyralufen-ethyl, there was no mortality even when they were treated at the highest dose required by the guidelines. The vapour pressure of pyraflufen-ethyl is very low as 1.6×10^{-8} Pa at 25°C and therefore it was not considered necessary to perform a short-term inhalation study.

B.5.3.3.2 90-day inhalation toxicity (rat) (Annex IIA 5.3.3)

No data, not necessary.

B.5.3.3.3 Percutaneous 28-day toxicity (rat) (Annex IIA 5.3.3)

For the assessment of operator exposure additional percutaneous studies may be useful, however this requirement is not compulsory.

B.5.3.3.4 Percutaneous 90-day toxicity (rat) (Annex IIA 5.3.3)

No data, not necessary.

B.5.3.4 Summary of short-term toxicity (Annex IIA 5.3)

Oral administration of pyraflufen-ethyl to rats or mice for 4 weeks resulted in the expression of diminished availability of vital hemoproteins : males were more sensitive and showed reduced body weight, reduced erythrocyte characteristics, increased plasma bilirubin indicative of mild hemolytic anemia associated with a compensatory response from the bone marrow (in rats) and from the spleen (extramedullary haematopoieisis in rats; increased weight in mice). Liver weight increase was associated with increase in AP, ALT, AST, suggesting liver dysfunction and liver necrosis. (See supplementary studies).

Oral administration to rats of pyraflufen-ethyl for 13 weeks confirmed the erythrocytes and liver as targets of toxicity. Minor renal effects were also reported.The NOAEL in this study was 5000 ppm (455 mg/kg bw/d).
Mice seems to be the most sensitive species : at 523.7 mg/kg bw/day, anemia and liver toxicity was reported. In this study a NOAEL of 20 mg/kg bw /d was retained. This value was also the lowest NOAEL in short-term toxicity studies and therefore used for the calculation of the AOEL.
Dogs did not shown any toxic effect after 13 or 52 week administration.

Table B.5.3.4-1 : Summary of short term-toxicity of pyraflufen-ethyl :

Type of test	Compound and test substance purity	Results			References
		NOAEL (mg/kg bw /day)	LOAEL (mg/kg bw/day)	Critical endpoints	
Rat, oral, 28 day	B.n°.3AM0011N 96.8 %	223.2	2296.4	bwî; liver weight ì; AP and ALT ì; ì bilirubin and cholesterol ; extramedullary hematopoieisis; symptoms of haemolytic anemia	Broadmedow, 1994a
Mice, oral, 28 day	B.n°.3AM0019P 97.6 %	<441.8	441.8	bwî; î RBC; îHb; î triglycerides	Takahashi, 1994
Rat, oral, 90 day	B.n°.3AM0011N 96.8 %	455.5	1489.4	bwî; altered blood parameters; ì spleen and kidney weight; liver hepatocytic hypertrophy; ìserum AP, ALT, AST,β-glob, îα-glob, albumin, glucose	Broadmeadow, 1994b
Mice, oral, 90 day	b.n°.4AM0021D: 97.6%; b.n°. 4 AM0023D, 98%	**20**	98	histopathological liver alterations	Kuwahara, 1996
Dog, oral, 90 day	97%; b. n° 4AM0024D	>1000	-	-	Broadmeadow, 1996a
Dog, oral,1 year	97.3%; b. n°. EN31653 ; 5AM0025D, 97.7% used up to week 51 and b.n°.5AM0026D	> 1000	-	-	Broadmeadow, 1996b
Rabbit, dermal, 21 day	no data				

B.5.4 Genotoxicity (Annex IIA 5.4)

B.5.4.1 In vitro genotoxicity testing (Annex IIA 5.4.1)

B.5.4.1.1 Gene mutation test in bacterial cells (Annex IIA 5.4.1)

- *Salmonella typhimurium* and *Escherichia coli* /mammalian-microsome mutagenicity test (May, 1994)

Findings: in the experiments performed with and without metabolic activation, treatment of strains TA98, TA100, TA1538, TA1535, TA1537 and WP2uvrA with pyraflufen-ethyl did not lead to an increase in the incidence of either histidine or tryptophan-prototrophic mutants in comparison with the negative control. In the confirmatory experiment, negative results were confirmed.

Conclusions:
Pyraflufen-ethyl did not induce reverse mutations in *S.typhimurium* or *E.coli*.

Guidelines:
Experimental protocol in compliance with test method B.14, Annex V, directive 92/69/EEC or directive 84/449/EEC or OECD guideline 471 (1983).
GLP :
Yes (no attest of competent authority)
Material and methods:
Pyraflufen-ethyl (97.6% ; b.n°.4AM0021D) dissolved in DMSO was tested for mutagenicity towards *S.typhimurium* TA 98, TA 100, TA 1538, TA 1535, TA 1537 and *E.coli* WP2 uvrA with and without liver S9 from Aroclor 1254 pretreated rats.
A preliminary toxicity test was performed with 8 concentrations from 2.5µg to 5 mg/plate with or without S9 mix (1.5 ml S9/ 8.5 ml mix). The assay was performed using the classical plate incorporation method using 156.3, 312.5, 625, 1250, 2500 or 5000 µg/plate. Results were confirmed in a second, independent experiment.
Indirect mutagens used as positive control : 2-aminoanthracene and benzo(a)pyrene.
Direct mutagens used as positive controls : Sodium azide, 9-aminoacridine and N-ethyl-N'-nitro-N-nitrosoguanidine..
The study is performed in good experimental conditions ; the acceptance and evaluation criterias are well defined and appropriate. Positive controls gave the expected results.
The study is accepted.

B.5.4.1.2 Gene mutation test in mammalian cells (Annex IIA 5.4.1)

- Mouse lymphoma cell line L5178Y/TK$^{+/-}$ (Lloyd,1994)

Findings:
In the preliminary toxicity test, there was no evidence of toxicity at concentrations up to and including 40.63 µg/ml without S9 mix and up to and including 81.25µg/ml with S9 mix.
Mutation assay: in the absence of S9 mix, cultures exposed to pyraflufen-ethyl showed no practical increase in TK +/- mutant colony number; no significantly increased mutant frequencies (per 10^5 survivors), compared to the solvent control, were apparent in either series of mutation assay. In the presence of S9 mix, no practical increase in mutant colony numbers or mutant frequencies over solvent control values were observed in the first mutation assay. However, in the second mutation assay, there was a dose-related increase in mutant colony numbers, compared to the solvent control cultures, and a corresponding increase in mutation frequency. These increases were observed at concentrations which were not highly toxic to mouse lymphoma cells.

Conclusion:
Pyraflufen-ethyl showed no mutagenic activity in the absence of metabolic activation system ; some evidence of mutagenic activity was shown in the presence of metabolic activation.

Comment :
As forward mutation study showed a slight positive evidence in the presence of metabolic activation, it was required from the notifier to confirm these results in a new study.

Guidelines:

Experimental protocol in compliance with test method B, Annex V, directive 87/302/EEC or OECD guideline 476 (1984).
GLP :
Yes; no attest of competent authority.
Material and methods:
Pyraflufen-ethyl (97.6% ;b.n°.4AM0021D) disolved in DMSO was first tested in a cytotoxicity test to establish a dose-range for the assay at 5.08, 10.16, 20.31, 40.63, 81.25, 162.5, 325, 650, 1300 and 2600 µg/ml with and without S9 mix. A solvent control was included.
Two main mutagenicity assay were conducted: pyraflufen-ethyl dissolved in DMSO (1% final concentration) was tested at 10, 20, 40, 60 and 80 µg/ml without S9 mix and 20, 40, 80, 120 and 160 µg/ml with S9 mix (rat liver S9 from Aroclor1254 pretreated rats) in the first experiment. The second experiment was realized with 20, 40, 60, 80 and 100 µg/ml without S9 mix and 40, 80, 120, 160, and 200 µg/ml with S9 mix.These concentrations were selected as pyraflufen-ethyl was proved to be less toxic than expected in the first mutation assay.
Ethylmethanesulfonate and DMBA were used as positive controls.
The study is accepted.

- Mouse lymphoma cell line L5178Y/TK$^{+/-}$ (Tanaka, 1998)

Findings:
In the preliminary toxicity test, cytotoxicity was apparent at 60 µg/ml without S9 mix and at 450 µg/ml with S9 mix. In both cases, 20%relative suspension growth was observed. Therefore, the mutation assay was performed at max 50 µg/ml without S9 mix and at maximum 350 µg/ml with S9mix.
Mutation assay: in the absence of S9 mix, cultures exposed to pyraflufen-ethyl showed no increase in TK +/- mutant colony number; no significantly increased mutant frequencies (per 10^5 survivors), compared to the solvent control, were apparent in either series of mutation assay.
In the presence of S9 mix, no increase in mutant colony numbers or mutant frequencies over solvent control values were observed in the 2 mutation assas..

Conclusion:
Pyraflufen-ethyl showed no mutagenic activity in these experimental conditions.

Guidelines:
Experimental protocol in compliance with test method B, Annex V, directive 87/302/EEC or OECD guideline 476 (1984).
GLP :
Yes; no attest of competent authority.
Material and methods:
Pyraflufen-ethyl (97.0% ;b.n°.4AM0021D) disolved in DMSO was first tested in a cytotoxicity test to establish a dose-range for the assay at 7.8 to 2000 µg/ml with and without S9 mix. A solvent control was included.
Two main mutagenicity assay were conducted: pyraflufen-ethyldissolved in DMSO (1% final concentration) was tested at 10, 20,30, 40, 50 µg/ml without S9 mix and 150, 200, 250, 300 and 350 µg/ml with S9 mix (rat liver S9 from Aroclor1254 pretreated rats) in the first and second experiment.
Methylmethanesulfonate and cyclophosphamide were used as positive controls.
The study is accepted.

B.5.4.1.3 *In vitro* chromosome aberration assay (Annex IIA 5.4.1)

- chromosome aberration assay in human peripheral lymphocytes (Dance, 1994)

Findings:
In the preliminary test, precipitation was apparent in all cultures treated with 2600 µg/ml. At this dose, in the absence of S9 mix, reduced mitotic activity of 31 and 30% were seen in cultures harvested at 19 and 43 hour sampling time respectively. In the presence of S9mix, at 19 hour sampling, reductions in mitotic activity of 26, 24, 12 and 17% were seen at 20.8, 104, 520, 2600 µg/ml respectively. At 43 hr, reductions of 16, 12, 24 and 8% were seen in cultures treated at 20.8, 104, 520 and 2600 µg/ml respectively.
In the cytogenetic test, mitotic activity was reduced with and without S9mix at 19 hour.
No biologically or statistically significant increases in aberrant cell frequencies, over concurrent solvent control values, were seen in any treated group including or excluding gaps, at either sampling time, in the absence or presence of S9mix.
Conclusions:

In both experiments performed without and with metabolic activation, pyraflufen-ethyl did not increase the number of metaphases containing specific chromosomal aberrations.

Guidelines:
Experimental protocol in compliance with test method B.10, Annex V, directive 92/69/EEC or directive 84/449/EEC or OECD guideline 473 (1983).
GLP :
Yes (no attest of competent authority)
Material and methods:
pyraflufen-ethyl (97.6% ; b.n°.4AM0021D) dissolved in DMSO, with or without S9 from Aroclor 1254 pretreated rat livers, was investigatedin cultured human lymphocytes.
Positive controls : chlorambucil and cyclophosphamide. A preliminary toxicity test was performed at 4.16, 20.8, 104, 520, and 2600 µg/ml for 19 and 43 hours with and without S9mix.
The first cytogenetic test was performed with 650, 1300 and 2600 µg/ml, with or without S9mix., 19 and 43 hour sampling time.
The second test was preformed using only 19 hour sampling time with the same concentrations. One hundred metaphases were scored from each culture.
Expriments were performed in good conditions; positive controls gave the expected response.
The study is accepted.

B.5.4.1.4 Assay for other effects on the genetic material (Annex IIA 5.4.1)

-DNA repair test with *Bacillus subtilis* (Inagaki, 1994)

Findings:
Pyraflufen-ethyl at 343, 687, 1375, 2750, 5500 µg/disk, did not cause inhibitory zone in either the strain M45(rec^-) or the strain H17 (rec^+) even at the highest dose of 5500 µg/disk with or without S9mix. Positive controls gave the expected results.

Conclusion:
Pyraflufen-ethyl is negative in the DNA repair test under the conditions used in this experiment.

Guidelines:
Japanese MAFF guideline 59 Nohsan n°.4200(1985).
GLP :
Yes (no attest of competent authority)
Material and methods:
Pyraflufen-ethyl (98.1% ; batch n°.4AM0021D)was dissolved in DMSO. Recombination deficient strain M 45(rec) and wild strain H17 of *Bacillus subtilis* were incubated for the preparation of spores solution. The DNA repair test was performed as following: strain of bacteria as a spore suspension are poured on petri dishes with or without metabolic activation system (S9+ cofactors). A paper disk was impregnated with 20 µl of a solution of test substance or control substance and placed on the prepared spore agar plate. After 24 h incubation at 37°C, diameter of growth inhibition was measured. Each concentration was tested in triplicate. A result is positive when the difference in diameter of the growth inhibitory zones between the 2 strains was 5 mm or more and if this difference indicated significant dose-relationship.
Positive control: mitomycin C and 2-aminoanthracene. The study is accepted.

B.5.4.2 *In vivo* genotoxicity testing (somatic cells) (Annex IIA 5.4.2)

B.5.4.2.1 *In vivo* mammalian bone-marrow micronucleus test (Annex IIA 5.4.2)

-Mouse bone marrow micronucleus test (Edwards, 1994)

Findings:
Symptoms of toxicity were observed in 1 female receiving 2 times 1250 mg/kg bw and was killed. There was no evidence of toxicity to the bone marrow as no reduction in the ratio of polychromatic to mature erythrocytes was reported.The recorded incidence of micronuclei per 1000 mature erythrocytes varied between 0.0 and 4.0 throughout groups dosed on one occasion. For animals dosed on one occasion, the ratio of polychromatic to mature erythrocytes in the vehicle control group and groups given pyraflufen-ethyl, killed 24 h later was 0.9 in each case. The ratio for vehicle control and top dose was 1.0, 48 h after dosage reaching 0.9 for vehicle control and 0.8 for treated group 72 hour later.
For animals dosed 2 times, the ratio was 0.9 for solvent control and 0.9 and 0.8 at 2500 and 5000 mg/kg bw in the dosed groups.

Comment:
Heme synthesis occurs in bone marrow and liver where pyraflufen-ethyl acts as inhibitor on the oxidation of protoporphyrinogen.From the long term studies it appeared that bone marrow was relatively resistent to the toxic action of pyraflufen-ethyl. The mouse appeared to be the most sensitive species to the toxic effects of pyraflufen-ethyl and the fact that 1 animal showed symptoms of toxicity suggests that toxic dose was reached in this study.

Conclusion:
Under the given experimental conditions no evidence for clastogenic or other effects was obtained in mice treated with pyraflufen-ethyl.

Guidelines:
Experimental protocol in compliance with test method B12, Annex V, directive 84/449/EEC or OECD guideline 474 (1983).
GLP :
Yes, no attest of competent authority.
Material and methods:
In a preliminary toxicity test 2 mice/sex/dose received a single oral dose of pyraflufen-ethyl (97.6% ; b.n°. 4AM0021D) at 625, 1250, 2500 and 5000 mg/kg bw in corn oil. A second group received two doses at 24 h interval. All surviving animals were killed 72 h after the final treatment.
In the main study, 5 mice/sex/dose received orally, one or two times at 24 h interval, 1250, 2500 or 5000 mg/kg bw. Animals were killed 24 h after the final treatment. Some of the animals receiving a single dose were sacrificed 48 or 72 h after treatment. Chlorambucil was used as positive control. The study is accepted.

B.5.4.2.2 In vivo unscheduled DNA synthesis in mammalian cells (Annex IIA 5.4.2)

- Rat liver DNA repair (UDS) test in vivo, oral administration, 600 and 2000 mg/kg bw (Gant and Proudlock, 1998)

Findings:
A preliminary test was performed in which 4 rats were exposed to 2000 mg/kg bw pyraflufen-ethyl. As no toxic signs were obtained at 2000 mg/kg bw, this level was chosen as the high dose, which is the maximum dose level recommended by the OECD guidelines.
DNA repair test: no mortality or clinical signs were obtained. Rats treated with pyraflufen-ethyl did not show any significant increase in the gross or net nuclear grain count at any dose level at either the 2 or 14 hour expression time.

Conclusion:
Pyraflufen-ethyl has not shown any evidence of causing DNA damage in rat liver in this *in vivo* test system.

Guideline :
Experimental protocol in compliance with OECD guideline TG 486.
GLP :
Yes
Material and methods :
4 Sprague-Dawley male rats/dose received a single oral dose of pyraflufen-ethyl (99% ; b.n°.6AM00381)at 600 or 2000 mg/kg bw. A concurrent negative control was the vehicle (aqueous 1% w/v methyl cellulose) and positive control was dimethylnitrosamine at 4 mg/kg or 2 AAF at 50 mg/kg. Hepatocytes were isolated at 2 or 14 h after exposure. 150 hepatocytes were scored.
Expriments were performed in good conditions; positive controls gave the expected response.
The study is accepted.

B.5.4.3 *In vivo* studies in germ cells (Annex IIA 5.4.3)

No data. Not necessary.

B.5.4.4 Summary of genotoxicity (Annex IIA 5.4)

Genotoxicity of pyraflufen-ethyl was tested in a variety of different tests covering both, eukaryotes and prokaryotes *in vivo* and *in vitro*.
The aspect of mutagenicity is considered to be adequatly investigated.
While no point mutations were observed in bacterial cells, positive results were obtained in mouse lymphoma cells in the presence of metabolic activation. As the forward mutation study showed a slight positive evidence in the presence of metabolic activation, it was required from the notifier to confirm these results in a new study. In a second test conducted using lymphoma cells a clear negative response was observed.
Pyraflufen-ethyl was negative in the DNA repair test using the *Bacillus subtilis* rec assay as indicator, and did not cause DNA damage in rat liver *in vivo*.
Pyraflufen-ethyl did not induce clastogenic or other damage *in vivo* in mice.

In conclusion, pyraflufen-ethyl is not genotoxic.

Table B.5.4.4-1 : Summary of genotoxicity of pyraflufen-ethyl

Type of test Cell/Test species	**Test substance; purity**	**Conditions**	**Results**	**References**
***In vitro* gene mutation test**				
Salmonella and *Escherichia coli* / mammalian microsome test	pyraflufen-ethyl: 97.6%; b.n°.4AM0021D	TA98, 100, 1535, 1537, 1538 and WP2uvrA +/- S9 mix, DMSO; 156.3, 312.5, 625, 1250, 2500 and 5000 µg/plate	negative	May, 1994
mouse lymphoma cells L5178Y TK+/-	pyraflufen-ethyl: 97.6%; b.n°4AM0021D	- S9mix:10, 20, 40, 60 and 80 µg/ml +S9mix: 20, 40, 80, 120 and 160 µg/ml ; DMSO	-S9mix: negative + S9mix: positive	Lloyd, 1994
mouse lymphoma cells L5178Y TK+/-	pyraflufen-ethyl: 97.0%; b.n°4AM0021D	- S9mix:10, 20, 40, 50 µg/ml +S9mix: 150,200, 250, 300 and 350 µg/ml ; DMSO	negative	Tanaka, 1998
***In vitro* chromosome assays**				
chromosome aberrations in human lymphocytes	pyraflufen-ethyl: 97.6% b.n°4AM0021D	+/- S9mix : 650, 1300 and 2600 µg/ml	negative	Dance, 1994
***In vitro* DNA repair assays**				
rec assay, *Bacillus subtilis*	pyraflufen-ethyl: 98.1% b.n°4AM0021D	+/- S9 mix; 343, 687, 1375, 2750, 5500 µg/disk	negative	Inagaki, 1994
***In vivo* genotoxicity test**				
UDS in rat liver	pyraflufen-ethyl: 99% b.n°.6AM00381	600 or 2000 mg/kg bw	negative	Gant and Proudlock, 1998
micronucleus assay	pyraflufen-ethyl: 97.6% b.n°4AM0021D	mouse bone marrow : 1250, 2500, 5000 mg/kg bw, by gavage in corn oil	negative	Edwards, 1994

B.5.5 Long-term toxicity and carcinogenicity (Annex IIA 5.5)

B.5.5.1 Long-term (2 years) oral toxicity in the rat (Annex IIA 5.5)

-Rats, diet, 0, 80, 400, 2000, and 10000 ppm, 104 weeks (Patel, 1996)

Findings:
Main findings are described in table B.5.5.1-1 and table B.5.5.1-2. The MTD was reached.
Mortality: the statistical analyses of differential mortality did not reveal any trends indicating an effect of pyraflufen-ethyl.
Clinical signs: staining of the perigenital area, tail and tray paper beneath the cages as well as dark yellow coloration of the urine could be related to the increased porphyrin excretion.
Water intake was strongly increased for males and females at the top dose, and animals produced more urine than the controls suggesting a disturbance in water balance.
Haematology: In males given 10000 ppm there was a mild microcytic anaemia throughout the treatment period; females were lesser affected and only during the early part of the treatment period. The increased platelet numbers in males seen during the early phase of the study suggest a response by the bone marrow to an increased demand or usage. Since myeloid to erythroid ratios were not increased there is no evidence of bone marrow response.
The increased numbers of spherocytes observed in males given 10000 ppm indicate an impairment of the ability of the red cell to maintain its normal biconcave shape. Such changes may be due to minor damage to the cell membrane or an interference of the energy dependent processes involved. The increase in spleen weights in females may be associated with the inability of the red cell to maintain its normal shape as it is here that atypical erythrocytes are destroyed.
Clinical chemistry: the increase in ALT suggest an effect of treatment upon the liver.Increased urea suggest renal injury. The effects on albumin and globulin and on A/G ratio might be treatment-related but a clear dose-effect relationship is not observed.

Neoplastic findings:
In animals killed or dying during the treatment period, killed after 52 weeks or killed at terminal sacrifice, there were no neoplastic findings which were considered to be related to treatment with pyraflufen-ethyl.

Non-neoplastic findings:
After 52 weeks of treatment, changes were confined to kidneys (slight increased weight) and liver (periacinar hypertrophy of hepatocytes and bile duct hyperplasia at top dose) and considered to be related to the treatment.
In males given 10000 ppm that died or killed during the treatment period, there was a small increase in the incidence of transitional cell hyperplasia of the urinary bladder.

In animals killed after 104 weeks, these effects were confirmed.
In addition to an increase in kidney weight, there were several histopathological findings in the kidneys of animals receiving 10000 ppm that suggest an effect upon renal papilla which resulted in inflammation, necrosis and sloughing. Dilatation and hyperplasia of the collecting ducts, and transitional cell hyperplasia, were likely to be secondary to the changes in papilla. These changes were also evident among animals of the highest dietary concentration group which died or were killed prematurely. Increased plasma urea concentrations are likely due to renal effects.
Electron microscopy of the liver of animals killed after 52 or 104 weeks indicated the presence of electron-lucent vacuoles within the mitochondria of centriacinar and periacinar hepatocytes. This finding may occur in old animals ; however, the incidence in treated animals was high compared to the two incidences in control animals and therefore there is a clear association of the finding to treatment. The general appearance of the mitochondrial cristae and the presence of matrix granules suggested that there was no impairment of mitochondrial function.

Table B.5.5.1-1 : Main findings reported in rats after 104 week treatment with pyraflufen-ethyl.

Dose/ endpoint	**0 ppm**		**80 ppm**		**400 ppm**		**2000 ppm**		**10000 ppm**	
	_	_	_	_	_	_	_	_	_	_
Mortality (cumulative)										
week 0-52	2	1	2	3	3	2	4	1	2	1
week53-72	6	9	7	6	5	7	9	8	7	3
week73-90	12	24	16	18	19	25	21	25	18	24
week 91-104	24	33	25	35	30	39	33	33	29	32
Clinical signs: Perigenital staining								ì	ì	ì wk 22
Brown staining of tail										ì wk 20-68
Cage paper staining									ìwk 64	ì wk 64
body weight (%)			104	100	97	103	94	97	84	89
food intake (%)			100	100	99	100	100	100	94	101
water intake (ml/kg/day)	58	93	47	96	52	84	62	90	80	130
Ophtalmo.	No effects									
Haematology										
PCV						ì13%*			î16% **	
Hb						ì13%*			î16% **	
MCHC							î1%*			
MCV									î10% **	
MCH									î11% ***	
WBC								î28%*		
Platelets				î16%*				î23%**		
Presence of spherocytes few/several	2/0	0/0	0/1	0/0	1/0	0/0	2/0	0/0	3/2	0/0
slight/moderate anisocytosis	0/00	0/0	0/0	0/0	1/0	1/0	3/0	0/0	3/1	0/1
Clinical chemistry:										
ALT									ì90% *	
glucose						ì15%*		ì17%**		
urea									ì80% **	
albumin					î24% ***	ì9%*	î18% ***			
α1 globulin					ì30% *					
α2 globulin					ì16% *					
β globulin				î18%*	ì12% **					
A/G ratio			î13%*	ì22%*	î38%		î25%			

Dose/ endpoint	0 ppm		80 ppm		400 ppm		2000 ppm		10000 ppm	
	_	_	_	_	_	_	_	_	_	_
					***		***			
urinalysis										
volume				ì50%*					ì50%*	ì75%**
pH				ì**	î**		î**	ì**		ì**
specific gravity			ì*	î*	ì**		ì**	î*		î***
Organ weights										
kidneys (a)							ì26%*		ì20%*	
spleen										(ì22%)
liver(a)					ì21% **				ì14%*	
testes(a)									ì39% **	

Statistically significant ì or î at : *p<0.05; ** p<0.01; *** p<0.001 according to Behren's-Fisher's test. () : not statistically significant ; (A) : absolute organ weight.

Table B.5.5.1.-2 : Macropathology and histopathology in animals treated with pyraflufen-ethyl

Dose/ endpoint	0 ppm		80 ppm		400 ppm		2000 ppm		10000 ppm	
	_	_	_	_	_	_	_	_	_	_
n°.examined week 104	21	15	25	15	19	11	17	17	20	18
abnormal urinary bladder content	0	0	0	0	0	0	0	0	4*	0
Electron microscopy of the liver: 3 animals										
week 52: electron-lucent vacuoles in centriacinar hepatocytes mito.								1/3	2/3	1/3
electron- lucent vacuoles in periacinar hepatocytes mito.				1/3			3/3	1/3	2/3	
week 104: electron-lucent vacuoles in centriacinar hepatocytes mito.	1/3	1/3			1/3	2/3	all animals		all animals	
Neoplastic findings in animals killed or dying during treatment:										
n° animals	29	35	25	35	31	39	33	33	30	32
mammary fibroadenoma(B)	0	20	1	18	1	23	0	26*	0	16
pituitary adenoma(B)	13	21	12	31*	17	30	11	20	11	22
Neoplastic findings in animals killed after 104 weeks:										
n° examined	21	15	25	15	19	11	17	17	20	18
	no effects									

Dose/ endpoint	**0 ppm**		**80 ppm**		**400 ppm**		**2000 ppm**		**10000 ppm**	
	_	_	_	_	_	_	_	_	_	_
Non-neoplastic findings for all animals : 50 animals										
Kidneys:transitional cell hyperplasia	6	13	8	7	7	5	3	7	20**	32***
papillary transitional cell hyperplasia	1	3	1	3	3	4	2	2	6	12*
papillary necrosis/sloughing	0	0	1	0	1	0	0	1	16***	16***
acute papillitis	0	0	0	0	0	0	0	1	11***	8**
collecting ducts dilatation/ hyperplasia	0	0	0	0	0	0	0	2	4	12***
dilated cortical tubules	0	0	0	0	0	0	0	0	4	10**
liver:										
bile duct hyperplasia	18	9	16	6	12	7	15	6	40***	36***
Lungs:										
vascular mineralisation	24	14	26	19	27	27*	26	24	17	17
Urinary bladder:										
transitional cell hyperplasia	0	0	3	0	2	0	1	1	6*	0

Statistically significant ì or î at : *p<0.05; ** p<0.01; *** p<0.001 according to Behren's-Fisher's test. (a) : absolute organ weight.

Conclusion:
NOAEL = 400 ppm = 20 mg/kg bw/d
Pyraflufen-ethyl was not carcinogenic under the conditions of the study.

Guidelines:
Experimental protocol not fully in compliance with test method B, annex V, directive 87/302/EEC or OECD guideline 453 (1981).
Deviation from official protocol: in this 24 mounth study, mortality was higher than 50% in the control. Therefore, negative results are not acceptable in these conditions. Ornithine decarboxylase was not measured.
GLP:
Yes (no attest of competent authority)
Material and methods:
50 rats (CD-Sprague-Dawley)/sex/dose were fed diets containing pyraflufen-ethyl (From week 1-17 : 97.6%, b.n°.3AM0019P: week 18-75 ; 97.6%, b.n°.4AM0021D; from week 76 onwards : b.n° 5AM0026D; 97.7%) at concentrations of 0,80, 400, 2000 or 10000 ppm.An additional group of 20 animals per dose were sacrified after 52 weeks and surviving animals were killed at 104 weeks.
Achieved dosages:
For males : 3.4; 17.2; 86.7; 468.1 mg/kg bw/d
For females : 4.4; 21.8; 111.5 and 578.5 mg/kg bw/d
The study is accepted.

B.5.5.2 Carcinogenicity study in the rat (Annex IIA 5.5)

See point B.5.5.1.

B.5.5.3 Carcinogenicity study in the mouse (Annex IIA 5.5)

- Mice, diet, 0, 200, 1000 or 5000 ppm, 78 week (Kuwahara, 1996)

Findings:
Main findings are described in table B.5.5.3-1 and table B.5.5.3-2.
Mortality was significantly increased in males (week 76 to 78) and females (week 47 to 51 and weeks 58 to 65) at 1000 ppm and in females treated with 200 ppm during weeks 47, 48, and 65.
Increase of opacity of the eye at the top dose was not associated with histopathological changes and therefore considered of no toxicological significance.
At the top dose, microscopic and macroscopic analysis showed that liver was the target organ and mice presented a significantly increased incidence of benign hepatocellular adenoma. Liver toxicity, observed at 1000 ppm was characterized by several histopathological lesions.The degree of damage of hepatocytes in females at 1000 ppm was not so severe as much as inducing cell proliferation or neoplastic changes.
These effects were not observed at 200 ppm.

Table B.5.5.3-1: Carcinogenicity study of pyraflufen-ethyl in mice.

Endpoints/ dose	**0 ppm**		**200 ppm**		**1000 ppm**		**5000 ppm**	
	_	_	_	_	_	_	_	_
Mortality /50 animals	20	13	17	16	32	16	18	15
Clinical signs			penis protrusion	hair loss	distended abdomen; mass of skin; soiled fur		eye opacity	
Body weight			no effect					
Food consumption			no effect					
Hematology								
lymphocytes							ì	
neutrophiles			ì				î	
Macroscopic findings in surviving animals:								
No mice examined	30	37	33	34	18	33	31	35
Liver								
-pale/accentuated lobular pattern	0/0	0/1	0/0	0/0	0/0	0/0	1/1	4/3
-coarse surface	0	0	2	0	0	0	5*	0
-spots	1	1	2	3	2	2	9**	9**
-masses	16	2	11	0	16*	3	25**	16**
Kidneys cysts	0	4	2	4	0	4	5*	1
Testis atrophy	0	-	0	-	3*	-	1	-
Macroscopic findings in decedents:								
No mice examined	20	13	17	16	32	16	19	15

Endpoints/ dose	0 ppm		200 ppm		1000 ppm		5000 ppm	
	_	_	_	_	_	_	_	_
Stomach with black content :	0	0	0	0	0	0	4	1
liver: masses	2	3	5	2	12*	0	11*	4
lymph nodes enlargement	3	0	3	4	6	3	5	5*
Organ weight:								
liver (a)/(r)					ì57%/		ì73%/ì60%	ì18%/

* and ** significantly different from control at 5 and 1% level of probability.
Statistically significant î or ì. a : absolute; r= relative.

Table B.5.5.3-2.: histopathological findings in mice treated with pyraflufen-ethyl for 78 weeks.

Endpoints/dose	0 ppm		200 ppm		1000 ppm		5000 ppm	
	_	_	_	_	_	_	_	_
Neoplastic lesions /60 mice								
liver: hepatocel. adenoma(B)	16 (3D, 13S)	1 (1S)	12 (2D,10 S)	0	24 (10D,14S*)	1(1S)	31** (9D,22S*)	16** (3D,13S)
carcinoma (M)	1	0	1	0	2	0	1	1
Non neoplastic lesions /60 mice								
focal hepatocel. necrosis	4	8	4	11	10	11	17**	8
centrilobular hepatocel. swelling	1	0	3	1	20**	9**	22**	47**
foci of cellular alterations (acidophilic)	7	2	6	1	21**	2	37**	12**
foci of cellular alterations (clear cell foci)	0	0	1	0	7**	0	26**	5*
hepatocel. vacuolation	0	0	2	0	7**	3	16**	15**
ì brown pigment depot of Kupffer cells	1	0	3	2	19**	3	35**	21**
micro-granuloma	22	15	17	24	30	20	40**	42**
single cell necrosis	2	0	2	1	3	5	3	10**
interstitial fibrosis	0	0	1	1	3	3	3*	6*
Adreno- cortico-medullary junction:								
ì brown pigment depots	6	1	1	3	8	3	18**	12**

* and ** significantly different from control at 5 and 1% level of probability; S surviving; D: decedent; B: benign; M : malignant.

Conclusion:
Pyraflufen-ethyl induces benign hepatocellular adenomas in liver mice at the top dose.
NOAEL = 200 ppm =20 mg/kg bw/d
Guidelines:
Experimental protocol in compliance with test method B, annex V, directive 87/302/EEC.
GLP :
Yes (no attest of competent authority)
Material and methods:
50 ICR (Crj:CD-1) mice/sex/dose received pyraflufen-ethyl (b.n°.4AM0021D: 97.6%; b.n°. 4 AM0023D, 98%) in the diet at 0, 200, 1000, and 5000 ppm, over a period of 78 weeks. Additional groups of 10 males and 10 females for interim sacrifice after treatment for 13 weeks were allocated at each dose.
Achieved intake :
Male: 0, 21, 109.7, and 546.8 mg/kg bw/d
female :0, 19.6, 98.3, and 523.7 mg/kg bw/d
The study is accepted.

B.5.5.4 Mechanism of action and supporting data (Annex IIA 5.5)

- Effect of pyraflufen-ethyl on the proliferative activity of mice hepatocytes (Kuwahara, 1997)

It is possible to detect cell division as pre-cancerous change by use of an antibody against proliferative cell nuclear antigen (PCNA).

Findings:
At the top dose, males and females showed significantly higher values of proliferative cell nuclear antigen (PCNA) labeling index after 13 and 78 weeks treatment. This effect was also observed in males at 1000 ppm and in females after 13 weeks (Table B.5.5.4-1).

Table. B.5.5.4-1. PCNA labeling indices in the liver of mice examined at 13 week interim kill and terminal kill after 78 weeks f treatment (% of control).

Dose	**200 ppm**		**1000 ppm**		**5000 ppm**	
	_	_	_	_	_	_
Week 13	58	70	317*	490*	475**	1150**
Week 78	400	220	1250**	780	1810**	1810**

* and ** Significantly different from control at 5% or 1% respectively (Dunnett's multiple comparison test)

Conclusion :
The NOAEL in this study after 13 or 78 weeks is 200 ppm as liver regeneration is suggested to occur at 1000 and 5000 ppm..

Material and methods:
Tissue sections of the liver from 8 male and female ICR(Crj:CD-1), sacrificed at weeks 13 and 78 in the oncogenicity study (described under B.5.5.3) were examined. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) on liver sections was investigated by the Avidin-biotinylated peroxidase complex method with anti-PCNA. Tissue sections of duodenum were used as positive samples.

B.5.5.5 Summary of long-term toxicity and carcinogenicity (Annex IIA 5.5)

The long-term studies performed in rat and mice confirmed that toxic effects of pyraflufen-ethyl are consecutive to its mechanism of action by decreasing the activity of the enzyme protoporphyrinogen oxidase located in the liver mitochondria, causing porphyrin accumulation consecutive to defective haemoglobin and cytochromes synthesis. In both long-term studies, target organs were blood, liver and kidney as also observed in the short-term toxicity studies. However, porphyrin acccumulation in liver was not measured.
In rats, chronic oral administration induces microcytic anemia. In kidneys, renal papilla were affected. Cytotoxicity observed in liver was probably consecutive to the oxidative stress induced by an increase in liver porphyrin which is characterized in rats, at 10000 ppm, by bile duct hyperplasia, electron-lucent vacuoles in hepatocytic mitochondria wtihout impairment of mitochondrial function and in mice, at 1000 ppm, by liver swelling, cytoplasmic vacuolation, pigment accumulation in Kupfer cells, foci of cellular alterations, liver hyperplasia (PCNA) and benign hepatocellular adenomas (at 5000 ppm). No adverse effect level for proliferative activity of hepatocytes is 200 ppm. Pyraflufen-ethyl was not carcinogenic in the rat study.
From both rat and mice studies, a NOAEL of 20 mg/kg bw/day is provisionally acceptable waiting for a NOAEL based on porphyrin accumulation in liver in mice.
It is concluded that the increased hepatocellular adenomas in the high dose group of the mouse carcinogenicity study might be induced through the non genotoxic/cytotoxic mode of action of pyraflufen-ethyl.
From the open literature, two retrospective studies and one case-control study have suggested an association between primary liver cancer and acute hepatic porphyrias and according to the authors, acute hepatic porphyria should be considered as a rare cause of hepatocellular carcinoma (Andant et al., 1998).

Table B.5.5.5-1 : Summary of long-term toxicity and carcinogenicity of pyraflufen-ethyl

Type of test Test species	**Test substance purity**	**Results**			**References**
		NOAEL (mg/kg bw/ day)	**LOAEL (mg/kg bw/day)**	**Critical endpoints**	
Rat, 104 week	From week 1-17 : 97.6%, b.n°.3AM0019P: week 18-75 ; 97.6%, b.n°.4AM0021D; from week 76 onwards : b.n° 5AM0026D; 97.7%	20	87	slight anisocytosis, ì kidney weight; electron lucent vacuoles in hepatocytic mitochondria	**Patel, 1996**
Mice, diet, 78 week	b.n°.4AM0021D: 97.6%; b.n°. 4 AM0023D, 98%	20	98	liver: histopahtological lesions	**Kuwahara, 1996**

B.5.6 Reproductive toxicity (Annex IIA 5.6)

B.5.6.1.1 Two generation reproductive toxicity in the rat (Annex IIA 5.6.1)

- rat, diet, 0, 100, 1000, 10000 ppm, 2 generation study (Fujii, 1996)

Findings:
- *Adult data :* Main adult data are reported in Table B.5.6.1.1-1.
Treatment related adverse effects were observed in the 10000 ppm group for body weight, body weight gain, food consumption and pathology. The histopathological findings in liver and kidneys as well as alterations in the gross findings and organ weights were attributed to the test substance. The spleen, another target organ, was not considered to be affected by pyraflufen-ethyl since no changes were observed in the organ weight and gross and histopahtological findings. The decrease in adrenal weights of F1 parental animals were not considered to be toxicologically significant since the values of the concurrent control group were above or at higher points within the range of historical control data of the laboratory and no histopathological changes were observed in this organ.
Reproductive performances were not affected in F0 and F1 parental animals.

Offsprings (Table B. 5.6.1.1-2): no significant differences were found in the incidence of pups found dead, number delivered, sex ratio, and viability index and gross pathological findings. At the top dose, the body weights of both F1 and F2 pups showed a reduced bw during from day 14-21 of lactation. These effects are related to pyraflufen-ethyl.

Table B.5.6.1.1-1: 2-generation study in rats : adult data.

Dose/ endpoint	**0 ppm**	**100 ppm**	**1000 ppm**	**10000 ppm**
Mortality F0,F1	1	1	1 1	1
clinical signs		no compound related effects		
body weight			ìF0	îF0,F1 îF1wk1-7
body weight gain				îF0,F1 îF1wk1-5
Food intake		no compound related effect		
Reproductive data F0/F1:				
mating index		normal		
fertility index (%)	100/95.7	91.7/95.7	100/100	87.5/95.8
gestation index (%)	100/100	100/100	95.8/100	100/100
gestation (days)	22.2/22.2	22.3/22.3	22.1/22.3	22.2/22.3
no.implantation sites	15.5/16	14.6/16.7	15.3/16.2	14.5/15.6
no.pups delivered	14.4/14.8	12.9/14.5	14.5/15.1	13.4/13.3
sex ratio	0.50/0.46	0.54/0.49	0.51/0.51	0.53/0.45
Viability index				
lactation day 0	97/98.8	92.8/96.3	98/98.1	98.3/97.7
lactation day 4	97.9/98	96.9/98.6	99.1/98.8	99.2/97.9
lactation day 21	99.5/100	95.5/98.9	100/100	98.2/100
Necropsy findings:				
F0 liver : dark colored				20/24*** 20/24***
F1 liver : dark colored				22/24*** 21/24***
F0 kidney: dark colored				14/24***
F1 kidney: dark colored				21/24*** 15/24***
Organ weight				

Dose/ endpoint	**0 ppm**	**100 ppm**	**1000 ppm**	**10000 ppm**
	_ _	_ _	_ _	_ _
liver (a)				F0î* ìF0*
adrenals (a)				F1î** F1î**
kidneys (a)				F1î*** ìF0*
brain(r)				ìF1*
adrenals(r)				F1î**
kidneys(r)				ìF0*** ìF1*** ìF1***
liver(r)				ìF0** iF1**
seminal vesicle(r)				ìF0**
pituitary			F0î**	
Histopathological findings:				
liver: depots in Kupffer cells				F0:24***F1 :24*** F0:13*** F1:20***
single cell necrosis				F1:24*** F0:17*** F1:18***
infl.cell infiltration				F1:23*** F0:16*** F1:17***
centrilobular hepatocel. swelling				F0:13***
bile duct proliferation				F0:22*** F1:20***
kidney: loss of acido.body in prox.tubule				F0:22*** F1:17*** F1:23***
brown depot in prox.tubul.cells				F0:17*** F0:5* F1:20***

Statistically significant ì or î at : *p<0.05; ** p<0.01; *** p<0.001 according Fisher's exact test; Barlett's test, Kruskal-Wallis test, Dunnet-type or Scheffé-type test ; a : absolute and r : relative.

Table B.5.6.1.1-2 : Mean litter data in F1 and F2 rats pups

Endpoints/dose	**0 ppm**		**100 ppm**		**1000 ppm**		**10000 ppm**	
	_	_	_	_	_	_	_	_
Mortality during lactation F1/F2	11/6		19/14		8/8		6/9	
Body weight day 21F1/F2							î*/î***	î**/î***

Conclusion:
NOAEL reproduction toxicity >10000 ppm = 721 mg/kg bw/d.
NOAEL syst.toxicity =1000 ppm = 70.8 mg/kg bw/d

Guidelines:
Experimental protocol in compliance with method B, Annex V, directive 87/302/EEC or OECD guideline 416 (1983).
GLP :

Yes (no attest of competent authority)
Material and methods:
24 rats(Crj:CD Sprague-Dawley) /sex/dose were treated via diet with pyraflufen-ethyl (b.n°. 4AM0023D, 97.6%) for 2 successive generations at 0, 100, 1000 or 10000 ppm.
A preliminary study was performed using 30, 300, 3000 or 10000 ppm for 3 weeks prior to mating and throughout the subsequent breeding period until weaning of the F1 pups at the age of 3 weeks.
Converted dose were in F0 males : 0, 6.84, 70.8 and 721 mg/kg bw/d.
F 0 females: 0, 7.78, 80.1 and 813 mg/kg bw/d.
Converted dose were in F1 males : 0, 8.1, 82.3 and 844 mg/kg bw/d.
F 1 females: 0, 9.06, 91.2 and 901 mg/kg bw/d.
The study is accepted.

B.5.6.1.2 Supplementary studies (Annex IIA 5.6.1)

No data, not necessary.

B.5.6.2.1 Teratogenicity test by the oral route in the rat (Annex IIA 5.6.2)

- rat, 0, 100, 300, 1000 mg/kg bw/d, by gavage, from day 6 to day 15 (Burns, 1995a)

Findings:
Main results are reported in Table B.5.6.2.1-1. One female died consecutively to the dosing procedure. There were no treatment -related effects in females.
Litter responses: all females were pregnant; gravid uterine weight, number of corpora lutea, implantations, resorptions and viable young, the extent of pre-and postimplantation loss and foetal and placental weights were similar to those of the controls. Placental weights in animals which received 100 mg/kg/day were significantly higher than those of the controls. As this finding was not reflected in animals which received 300 or 1000 mg/kg bw/day, it was not considered to be related to maternal treatment.
Foetal evaluation: Macroscopic evaluation of foetuses at necropsy revealed a number of observations which were of types and occurred at incidences previously recorded in this strain of rat and not related to treatment. Skeletal and visceral examination revealed a small number of inter-group differences, the majority of these findings were of types and occurred at frequencies previously seen in this strain of rats at this laboratory and were considered not related to treatment.

Table B.5.6.2.1-1. Maternal and fetal data.

Endpoints	**Dose (mg/kg bw/day)**			
	0	**100**	**300**	**1000**
Maternal data:				
Number pregnant / number mated	22/22	22/22	22/22	21/22
Clinical signs: brown stainning on head			1	2
Body weight during gestation		not treatment related effect		
Water consumption		not treatment related effect		
Food consumption day 0-19		not treatment related effect		
Uterine examination		not treatment related effect		
placental weight (g)	0.52	0.59***	0.55	0.53
Fetal examination: **External malformation** **:** n° fetuses (n° litters)				
localised internal abdominal haemorrhage	1.3 (2)	0.6 (1)	4.1 (4)	4.4 (6)

Endpoints	Dose (mg/kg bw/day)			
	0	**100**	**300**	**1000**
blood on tongue, mouth, sinuses or nasopharynx	1.9 (3)	1.9 (3)	2.7 (3)	3.1 (3)
testis displaced	14.5 (9)	26.3 (14)	14.8 (11)	17.9 (10)
Skeletal anomalies: n° fetuses (n° litters)				
incomplete ossification of interparietal bone	6.9 (9)	18.7 (14)	21.1(12)	15 (12)
incomplete ossification of hyoid bone	2.9 (5)	5.3 (6)	4.8 (5)	4.8 (4)
Statistical key : One way analysis of variance and/or t-test; nested analysis of variance ; Mann-Whitney U test; Chi-squared test and Fisher's exact test. *** $p<0.001$				

Conclusion:
There was no evidence for maternal toxicity, embryotoxic or teratogenic potential.
NOAEL maternal tox ≥1000 mg/kg bw/d
NOAEL foetal toxicity and development ≥1 000 mg/kg bw/d.

Guidelines :
Experimental protocol in compliance with test method B, annex V, directive 87/302/EEC or OECD guideline 414 (1981)
GLP :
Yes (no attest of competent authority)
Material and methods:
22 pregnant female rats CD/dose received by gavage pyraflufen-ethyl (b.n°4AM0024D; 97.0%) in 0.5% (w/v) methylcellulose at 0, 100, 300, or 1000 mg/kg bw/d from day 6 through day 15 of gestation inclusive.Animals were killed on day 20.
The study is accepted.

B.5.6.2.2 Teratogenicity test by the oral route in the rabbit (Annex IIA 5.6.2)

- Rabbit, gavage, from day 6-19, at 0, 20, 60 or 150 mg/kg bw/d (Burns, 1996)

Findings :
Table B.5.6.2.2 -1 :Teratogenicity test by the oral route in the rabbit

Dosage (mg/kg bw/d)	0	20	60	150
Total number inseminated	15	15	15	15
Found death	0	0	1	0
Killed for human reasons	0	0	1	3
Killed *in extremis*	0	0	1	2
Not pregnant	0	1	2	1
Aborted	0	0	0	3
Total litter loss	1	0	0	0
Pregnant at term with viable young	14	14	10	6
Foetuses examined	125	114	80	35

Applicant rational :
The applicant considers this study as acceptable for the assessment and that no further study is necessary and argued that :

In the high dose group (150 mg/kg/day), 6 pregnant animals had viable young and 35 offsprings were obtained. Both number might be to small to evaluate the teratogenicity potential of pyraflufen-ethyl. At the dose of 60 mg/kg bw/day, 10 pregnant animals with viable young could be used for the evaluation of teratology, and 80 offspring were available because of no increase of foetal resorption and death of offspring. In the offspring of the 60 mg/kg bw/d, no

effect on body weight, macroscopic findings, findings following freehand serial sectioning of fetal heads and findings at skeletal examination were observed.

Consequently, it can be concluded that there were no effects on the foetal death, development and malformation even at the dose, at which some dams were died by the treatment. It was obvious that the 20 mg/kg bw/d was the NOAEL.
No clear treatment-related effects, except death, were observed in the study. The difference between the high and low doses was relatively small. From these reasons, it did not seem to be posssible to conduct another study at lower doses for the evaluation on the teratogenic potential without maternal death.

RMS conclusion :
The RMS considers that the study is not acceptable due to the high mortality observed at the top dose and another study at lower doses is necessary. The RMS thinks that this point must be discussed during the ECCO meeting.

Guidelines:
Experimental protocol not fully in compliance with test method B, annex v, directive 87/302/EEC or OECD guideline 414 (1981).
Deviation from official protocol: mortality at 60 and 150 mg/kg bw/d > 10% and not enough pregnant rabbits making the study not acceptable.
GLP :
Yes (no attest of competent authority)

Material, methods and findings:
15 female New Zealand rabbits (Harlan UK Ltd) received by gavage, pyraflufen-ethyl (97%, b.n° 4AM0024D)in 0.5% methylcellulose at 0, 20, 60 and 150 mg/kg bw/d from day 6 to 19 of gestation inclusive.
Five females which received 150 mg/kg bw/d were early decedents between day 16 and 24 of gestation ; 2 were killed *in extremis* and 3 for human reasons. Reduced food intake, body weight and faecal output. Gastro-intestinal tract was disturbed. Three other females aborted.
At 60 mg/kg bw/d, 1 was found dead and 2 other were early decedents. The same clinical signs were observed as at 150 mg/kg bw/d.
60 mg/kg bw/day seems to be higher than the MTD as 3/15 rabbits died.

B.5.6.3 Summary of reproductive toxicity and teratogenicity (Annex IIA 5.6)

In the two generation study, parental animals receiving pyraflufen-ethyl at the highest dose revealed a decreased body weight gain and pathological changes in liver and kidneys, and their offspring showed a decreased body weight gain during lactation period. However there were no abnormalities in the reproductive performance of parental animals and the reproductive index of offspring. Rats did not show any treatment-related changes in the teratology study.
Rabbits appeared to be strongly sensitive to the toxic effects of pyraflufen-ethyl. Due to the high mortality and insufficient number of pregnant rabbits at the 2 top doses, the study was not accepted by the RMS.

Table B.5.6.3-1 : Summary of reproductive toxicity and teratogenicity of pyraflufen-ethyl

Type of test **Test species**	**Test substance purity**	**Results**			**References**
		NOAEL (mg/kg b w/ day)	**LOAEL (mg/kg b w /day)**	**Critical endpoints**	
rat, 2 generation study	b.n°. 4AM0023D, 97.6%	-reproduction tox > 721 mg/kg bw/d. -syst.tox.= 70.8 mg/kg bw/d	- -721mg/kg bw/d	- -îbw, kidney and liver toxicity	**Fujii, 1996**
rat, developmental	b.n°4AM0024 D; 97.0%	-maternal tox ≥1000 mg/kg bw/d - foetal toxicity, development ≥1 000 mg/kg bw/d.	-	-	**Burns, 1995a**
Rabbit, developmental	b.n°4AM0024 D	The study is not accepted by the RMS; a NOAEL is proposed by the notifier = 20 mg/kg bw/d			**Burns, 1996**

B.5.7 Delayed neurotoxicity (Annex IIA 5.7)

No data, not necessary.

B.5.8 Further toxicological studies (Annex IIA 5.8)

Further studies were performed in order to investigate:
1. The effect of pyraflufen-ethyl on porphyrin accumulation *in vivo* and *in vitro*.
2. Effects on hepatic drug metabolizing enzymes in mice liver, *in vivo* and *in vitro*.
3. Effects of pyraflufen-ethyl on lipid peroxidation, beta oxidation and catalase activities, 8-Oh dG production in mouse liver.
4. Investigation of liver injury in male mice.

1. Porphyrin accumulation in rats hepatocytes(primary culture) and in rats and in mice liver : 4 week in diet, recovery of 2 weeks (Nakatani, 1998)

Findings:
- *In vivo* study:
In mice, mortality reached 75% at the top dose at day 9.
After 1, 2 and 4 weeks treatment with pyraflufen-ethyl, porphyrin concentration increased strongly in liver, blood plasma and bone marrow dose-dependently. The highest concentrations were observed in kidney, liver, bone marrow and blood ; lower concentrations were noted in spleen, lung, pancreas, testis and adipose tissues while adrenals, Harderian glands and skin were not affected. In liver, concentration increased time-dependently (table B.5.8-1).
After a recovery period for 2 weeks, porphyrin concentration in liver, blood, spleen and kidney (5000 ppm) group still showed significantly higher levels compared to the control group, but decreased clearly from the levels of the treatment for 4 weeks. At 3000 ppm, except for blood, no statistically significant higher concentration of porphyrin was observed (table B.5.8-2).
In rats, at 2000 ppm onwards, concentration of porphyrin was the highest in kidney, bone marrow and liver ; lower concentrations were observed in spleen and erythrocytes. The concentrations of porphyrin did not increase strongly with duration of exposure(table B.5.8-3).
- *In vitro* study: As reported in table B.5.8-4, porphyrin accumulation is observed in rat hepatocytes and increased dose-dependently from 0.5 µM onwards.

Table B.5.8-1: porphyrin accumulation in mice tissues after 1, 2 or 4 week exposure to pyraflufen-ethyl.

Tissue/dose	**0**			**3000 ppm**			**5000 ppm**		
Week	**1**	**2**	**4**	**1**	**2**	**4**	**1**	**2**	**4**
Liver µg/g	0.46	0.45	0.69	2.56**	5.52**	19.41**	3.72**	4.91**	15.8**
Blood µg/ml	0.32	0.67	0.32	5.95*	5.41**	5.36**	6.85**	10.79**	14.72**
Plasma µg/ml	NM	0.43	0.78	NM	0.84	1.48	NM	2.02	2.53**
Spleen µg/g	NM	0.43	0.47	NM	4.94**	5.59**	NM	23.02**	10.7**
Kidney µg/g	NM	1.11	0.24	NM	24.85**	7.65**	NM	33.01**	6.07**
B.m. µg/10^9 cells	5.83	4.10	9.47	18.17**	26.33**	43.54**	41.10**	46.97**	34.2
Lung µg/g	NM	NM	0.19	NM	NM	1.61*	NM	NM	4.21**
Pancreas µg/g	NM	NM	0.07	NM	NM	0.63**	NM	NM	0.98**
Adrenal µg/g	NM	NM	0.89	NM	NM	6.14	NM	NM	12.12
Adipose tissue µg/g	NM	NM	0.10	NM	NM	0.34*	NM	NM	0.44**
Harderian glands µg/g	NM	NM	0.14	NM	NM	0.15	NM	NM	2.33
Testis µg/g	NM	NM	0.11	NM	NM	0.60**	NM	NM	1.22**
Skin µg/g	NM	NM	0.33	NM	NM	1.1	NM	NM	0.33
* $p<0.05$; ** $p<0.01$ vs 0 ppm ; NM not measured									

Table 5.8-2: Concentrations of porphyrin in mice tissues after 2 week recovery.

Tissue	**2 week recovery**		
Dose	**0**	**3000 ppm**	**5000 ppm**
Liver µg/g	1.02	1.18	1.64*
Blood µg/ml	0.23	1.21**	2.01**
Plasma µg/ml	0.51	0.60	0.28
Spleen µg/g	0.42	0.89	1.97
Kidney µg/g	0.24	0.39	0.58**
B.m. µg/10^9 cells	3.12	5.62	5.64
Lung µg/g	0.17	0.34	0.53**
Pancreas µg/g	0.12	0.12	0.17
Adrenal µg/g	0.08	0.04	0.01
Adipose tissue µg/g	0.15	0.05	0.14
Harderian glands µg/g	0.39	0.19	0.28
Testis µg/g	0.11	0.24	0.27
Skin µg/g	0.14	0.05	0.21
* p<0.05 ; ** p<0.01 vs 0 ppm			

Table B.5.8-3: porphyrin accumulation in rat tissues after 1, 2 or 4 week exposure to pyraflufen-ethyl.

Tissue/ dose	**0**			**400 ppm**			**2000 ppm**			**10000 ppm**		
Week	**1**	**2**	**4**	**1**	**2**	**4**	**1**	**2**	**4**	**1**	**2**	**4**
Liver µg/g	0.59	0.46	0.45	0.58	0.49	0.49	1.32**	1.42*	1.07**	8.77**	7**	10.7**
erythrocytes µg/10^{10} cells	0.12	0.19	0.13	0.14	0.23	0.11	0.2*	0.44**	0.17	3.29**	3.13**	4.79
Spleen µg/g	0.32	0.5	0.8	0.3	0.3	0.35**	0.4*	0.51	0.46	13.44**	13.9**	12.6**
Kidney µg/g	1.0	1.1	1.3	1.0	1.4	1.7*	NM	29.0**	13.91**	35.03**	40.0**	48.5**
B.m. µg/10^{10} cells	3.62	4.35	3.03	4.62	4.57	3.68	6.04*	9.9	4.85**	125**	400**	136**
* p<0.05 ; ** p<0.01 vs 0 ppm ; NM : not measured												

Table B.5.8-4: Effect of pyraflufen-ethyl on porphyrin concentration in rat hepatocytes primary cell culture.

Pyraflufen-ethyl concentration (µM)	**Porphyrin concentration (ng/mg protein)**
0.0	19.52±2.64
0.1	26.64±1.75
0.5	41.72±10.76*
2.5	74.46±7.57**
12.5	127.41±1.11**
62.5	165.62±0.62**
313	212.22±0.21**

* p<0.05 ; ** p < 0.01 vs 0 µM

Conclusion:
Oral administration of pyraflufen-ethyl to mice results in an accumulation of porphyrin in rat and mice tissues. Mice were more sensitive.
Cessation of pyraflufen-ethyl administration results in a normalization of porphyrin levels after 2 weeks.
From the *in vivo* study, a NOAEL<3000 ppm for the mice study and a NOAEL = 400 ppm for the rat study is acceptable.

Comment :
According to the open literature, photosensitivity and related dermopathy are the prominent problems caused by deficiency of uroporphyrinogen decarboxylase, or uroporphyrinogen III synthase or ferrochelatase. However, photosensitivity is also characteristic of protoporphyrinogen oxidase deficiency, known as *Variegate porphyria* which is preferentially classified as hepatic porphyria, because biochemical expresssions are especially prominent in liver. The definitive diagnostic tests for *variegate porphyria* is quantification of the protoporphyrinogen oxidase in hepatocytes, because of the occurrence of major expression in hepatocytes (Ellefson and Ford, 1996).

Material and methods:
- *In vitro* study in rat hepatocytes in culture:
Hepatocytes of a male Fischer 344 rat were treated with pyraflufen-ethyl, at 0.1-313 µM pyraflufen-ethyl (98.2%; lot n° 2AM-0001P), dissolved in DMSO for 48 h. Cells were harvested and porphyrin concentration was measured by fluorescence using Protoporphyrin IX as standard.

- *In vivo* studies :
- 5 male mice (ICR, Charles-River) received in the diet pyraflufen-ethyl (97.6% ; lot n° 4 AM 0021D) at 0, 3000, 5000 or 10000 ppm for 4 weeks. A 2 week recovery period was designed.
Organs / tissues examined : liver, blood, plasma, spleen, kidney, bone-marrow cells, lungs, pancreas, adrenals, adipose tissue, Harderian glands, testes and skin.
- 5 male rats (SD) received pyraflufen-ethyl (97.6% ; lot n° 4 AM0021D) in diet for 4 weeks at 0, 400, 2000 or 10000 ppm.
Organs/tissues examined : liver, erythrocytes, spleen, kidney and bone marrow cells.
Porphyrin measurement : tissues were homogenized in a mixture of ethyl acetate and acetic acid (2:1), equal volume of 0.5 N HCL was added and mixed.The lower layer was collected and fluorescence was measured on a spectrofluorometer using Protoporphyrin IX as standard or Coproporphyrin III-2 HCL.
Porphyrin was calculated as equivalent of protoporphyrin IX.
The study is accepted.

2. Effects of pyraflufen-ethyl on mice hepatic drug metabolising enzymes after a single or repeated oral administration (Amanuma, 1996)

Findings:
- Single dose administration: while relative liver weight and microsomal protein concentrations were not affected by a single acute dose of pyraflufen-ethyl, it appeared that the high dose was able to induce statistically significant reductions of cytochrome P450 dependent activities as well as a non significant reduction of cytochrome P 450 level. These effects were apparent 24 h after treatment and evolve to recovery after 48 h (Table B.5.8-5).
- After repeated oral administration, the dose of 5000 ppm clearly affected the liver as suggested by the increase of relative liver weight, a non significant decrease of cytochrome P 450 level associated with reduced activities of the enzymes dependent of cytochrome P450 (table B.5.8-6).

Table B.5.8-5. Effect of a single oral administration of pyraflufen-ethyl on some mice liver enzymes.

Endpoints		**pyraflufen-ethyl**					
single dose	Control range	**5000 mg/kg bw**			**10000 mg/kg bw**		
time of sacrifice (h)		**6**	**24**	**48**	**6**	**24**	**48**
Relative liver weight	5.61-6.05	5.54	6.08	5.88	5.31	6.07	6.19
microsomal proteins	11.84-15.64	17.47	12.74	12.63	15.26	13.93	13.92
Cytochrome P450 (nmol/mg prot.)	0.76-0.99	0.69	0.75	0.60	0.66	0.52	0.56
Ethoxyresorufin O-dealkylase (pmol/min/mg prot.) CYP 1A1/2	88.01-144.9	94.07*	77.34	82.32	86.81*	59.85	46.26**
Pentoxyresorufin O-dealkylase (pmol/min/mg prot.) CYP 2B1	79.53-84.83	56.26	80.39	71.06	74.85	63.10	46.75
Aminopyrine N-demethylase (nmol/min/mg prot.)	2.84-3.71	2.63	3.05	4.00	1.99	2.43**	2.72
Aniline hydroxylase (nmol/min/mg prot.) CYP 2E1	1.04-1.15	0.95	1.13	1.18	1.00	0.88**	1.01
Ethoxycoumarin-O-deethylase (nmol/min/mg prot.) CYP 2B1	0.66-0.74	0.52	0.54*	0.80	0.66	0.42**	0.61

** $p<0.01$; * $p<0.05$

Table B.5.8-6 : Effect of 28 day oral administration in diet of pyraflufen-ethyl on some liver cyt P450 dependent isoenzymes..

Endpoints	**Control**	**pyraflufen-ethyl**			**phenobarbital**
Treatment		**28 day**			**28 day**
Dose		**200 ppm**	**1000 ppm**	**5000 ppm**	**1200 ppm**
Relative liver weight (g)	5.76	5.73	6.14	7.01**	9.33**
microsomal proteins (mg/g)	13.06	10.62*	12.3	13.85	13.51
Cytochrome P450 (nmol/mgprot.)	0.68	0.47	0.52	0.38	1.69**
Ethoxyresorufin O-dealkylase (pmol/min/mg prot.)	135.07	166.24	122.83	56.17*	242.17
Pentoxyresorufin O-dealkylase (pmol/min/mg prot.)	89.09	108.54	95.15	45.74**	693.92**
Aminopyrine N-demethylase (nmol/min/mg prot.)	3.03	2.91	1.95	0.63**	6.94**
Aniline hydroxylase (nmol/min/mg prot.)	1.02	1.04	1.00	0.83**	1.33**
Ethoxycoumarin-O-deethylase	1.13	0.90	0.88	0.57**	2.08**

(nmol/min/mg prot.)					

** p<0.01; * p<0.05

Conclusion :

From the *in vivo* study, a single dose of 10 000 mg/kg bw is necessary to alter some cytochrome P450 isoenzymes. After 28 day administration, a dose of 5000 ppm (» 1000 mg/kg bw/d) inhibited EROD, PROD, ECOD, aniline hydroxylase and aminopyrine demethylase significantly while cyt P450 content was not significantly reduced. A NOAEL= 1000 ppm (» 200 mg/kg bw/d) can be defined.

- *In vitro* effects of pyraflufen-ethyl or its metabolite on mice liver cyt.P450 level and dependent activities (Amanuma, 1996)

Findings:

Control microsomes were incubated with pyraflufen-ethyl or its metabolite E-1. Both compound did not affect the level of cytochrome P-450. 2 Enzymatic activities were measured: while the statistical analysis did not reveal a significant reduction, it appeared that E.R.O.D. was decreased at 100 µg/ml in the presence of pyraflufen-ethyl and at 1000 µg/ml in the presence of E-1. Aminopyrine demethylase was also reduced at 1000 µg/ml of pyraflufen-ethyl or E-1 (table B.5.8-7).

Table B.5.8-7 : incubation of pyraflyfen-ethyl or E-1 in the presence of mice liver microsomes.

Endpoints	**Control**	**pyraflufen-ethyl**			**E-1**		
Dose (µg/ml)		**10**	**100**	**1000**	**10**	**100**	**1000**
Cytochrome P450 (nmol/mg prot.)	0.699	0.693	0.708	0.678	0.711	0.693	0.678
Ethoxyresorufin O-dealkylase (pmol/min/mg prot.)	40.62	41.12	24.07	-	41.62	40.62	29.08
Aminopyrine N-demethylase (nmol/min/mg prot.)	3.36	3.62	3.06	2.69	3.7	3.4	1.60

Conclusion :

Pyraflufen-ethyl and its main metabolite did not inhibit cytochrome P450 content ;some small effects were observed on EROD and aminopyrine demethylase activities. These data suggest that pyraflufen-ethyl is more effective inhibitor than its metabolite.

Material and methods :

Enzyme induction was studied in 5 male JCR mice treated with pyraflufen-ethyl (b.n°. 4AM0021D; purity 97.5%) in corn oil by single oral gavage at 0, 5000 and 10000 mg/kg bw and sacrificed after 6, 24 or 48 h, or in the diet at 0, 200, 1000 and 5000 ppm for 28 days. Liver was homogeneized and microsomal fractions were prepared. A control was prepared in parallel. Phenobarbital was used as positive control.

For *in vitro* assays of enzymes modulation, pyraflufen-ethyl or metabolite E1 were dissolved in DMSO ; concentrations in incubation media were from 10 to 1000 µg/ml incubation medium.Incubation time was not given.

The study is accepted.

3. Effect of pyraflufen-ethyl on lipid peroxidation, β-oxidation activity, catalase activity and 8-hydroxydeoxyguanosine production in mouse liver (Inagaki, 1998)

Findings:

The table B.5.8-8 shows that feeding mice with pyraflufen-ethyl during 7 days induced at doses as high as 5000 ppm an increased absolute an relative liver weight associated with an increased lipid peroxidation, increased beta oxidation activity suggesting an increase in hepatic peroxisome proliferation, and decreased catalase activity. At the top dose of 10 000 ppm, an increased oxidative DNA damage represented by an increase of 8-OH dG was observed.

Table B.5.8-8 : effect of 7 day administration of pyraflufen-ethyl on the liver markers of oxidative stress.

Dose/endpoint	**0**	**200 ppm**	**1000 ppm**	**5000 ppm**	**10000 ppm**
Body weight				(î 5%)	î 25%
Liver weight				ì38%	(î 17%)
Relative liver weight				ì46%	
lipid peroxide conc. (nmol/g)				ì220%	ì220%
β- oxidation (nmol/mg protein/min)				ì367%	
catalase (µmol/mg protein/min)				î 86%	
8-OH dG conc (/10^5 dG)					ì78%
ì or î : statistically significantly according to Dunnett test ; $p < 0.01$ () = not statistically significant					

Conclusion:
NOAEL = 1000 ppm (» 200 mg/kg bw/d)

Comment :
It appeared in this study that catalase, which is a ubiquitous heme protein catalyzing the dismutation of H_2O_2, is strongly inhibited at doses of 5000 ppm in the diet. This effect is consecutive to the mechanism of action of pyraflufen-ethyl. As catalase is one of the major antioxidant enzymes involved in the detoxification of H_2O_2, an inhibition of this enzyme may lead to an increased level of reactive oxygen species within the cell leading to lipid peroxidation and formation of 8-OH-dG. Moreover, the increased peroxisomal beta oxidation leads also to the production of H_2O_2. A variety of base oxidation products have been shown to result from the reaction of reactive oxygen species with DNA. The modified base 8-hydroxy-2'-deoxyguanosine (8-OH-dG) is one of the identified products of oxidative base damage and it has been used as an indicator of oxidative DNA damage (Ye and Bodell, 1996).
The pro-oxidant characteristics of pyraflufen-ethyl resulting from the inhibition of hemoprotein synthesis may contribute to the liver cell necrosis observed in mice.

Material and methods:
5 male ICR mice/dose received pyraflufen-ethyl (lot n°.94-T-0055 ; 97.5%) in their diet at 0, 200, 1000, 5000 or 10 000 ppm for 7 days. Animals were sacrificed and liver homogenized in NaCL 9‰. Malondialdehyde was measured as an indicator of lipid peroxidation.
β- oxidation was measured by increase of A340 nm meaning NADH production from palmitoyl-CoA ; catalase activity was measured by following disappearance of H_2O_2 at 240 nm and protein was measured using a Bio-Rad assay.
8-OH-dG: was measured in liver after extraction of DNA which was treated with nuclease P1 and alkaline phosphatase followed by a HPLC -EC detection analysis.
The study is accepted.

4. Investigation of liver injury caused by dietary administration of pyraflufen-ethyl in mice (Nakatani, 1994)

Findings:
Effects of pyraflufen-ethyl were investigated in male mice as a function of time (Table B.5.8.-9).
Mortality: was again observed at the top dose, reaching 100% mortality at week 2.
Body weight decreases were observed at 5000 ppm starting at week 2 and did not recovered completely after the recovery period.
Organ weight and *clinical chemistry*: increased relative liver weight was observed at 3000 ppm onwards, starting the first week of exposure and this effect had a tendency to decrease with time without full recovery. Liver weight

increase was followed by an increase in ALT at 3000 ppm. At 5000 ppm, AST, AP were also increased while total protein and albumin were decreased. After 2 week recovery, blood chemistry altered parameters were no more affected.

Liver histopathology (Table B.5.8-10) showed that at 3000 ppm, after 2 week exposure, hepatocellular necrosis occurred. This effect appeared earlier at 5000 ppm and while recovery was complete at 3000 ppm, some small liver cell necrosis was still present in 1 animal at 5000 ppm. Hepatocellular necrosis appeared as a single cell necrosis and was rarely accompanied with cell inflammation.

During exposure, mitosis was induced and was more severe at 3000 ppm than at 5000 ppm. This effect results from liver cell necrosis.

After recovery period, eosinophilic body in cytoplasm and phagocytosis of red blood cell in the hepatocytes disappeared. The hepatocellular necrosis, cell inflammatory foci, mitosis and hepatocellular hypertrophy recovered but not completely.

Table B.5.8-9 : 28 days subacute study of pyraflufen-ethyl in mice.

Endpoint/ Dose (ppm)	**0**				**3000**				**5000**				**10000**	
Week	1	2	4	6	1	2	4	6	1	2	4	6	1	2
Mortality :%													60	100
Body weight changes										î 12%	î 8%	î 7%	î 17%	-
Clinical chemistry:														
AST								rec		ì 132%	ì 132%	rec	ì 1500%	
ALT						ì 127%	ì 451%	rec		ì 711%	ì 833%	rec		
AP											ì 143%	rec		
Glucose						î37%	rec			î 45%	rec			
tot. Protein												î 6%	î 27%	
Albumin												î 8%	î 27%	
Liver weight: relative					ì 35%	ì19%	ì33%	ì20%	ì48%	ì28%	ì36%	ì18%	ì24%	
Necropsy:														
Liver: accentuated lobular pattern					5		5	4	3		5	5	5	
Liver : whitish spots						5	5			4	4		2	
Adipose tissue: edema around testis													3	
Urinary bladder: retention urine													10	
Gallbladder: retention reddish bile juice													2	

Lung: congestion												6

Week 6 = 4 week treatment + 2 week recovery ; rec = recovery

Table B.5.8-10 : histopathological findings in liver of mice after treatment with pyraflufen-ethyl

Dose/ endpoint	**0**				**3000 ppm**				**5000 ppm**				**10 000 ppm**
Week	**1**	**2**	**4**	**6**	**1**	**2**	**4**	**6**	**1**	**2**	**4**	**6**	**1**
cellular necrosis:													
1cell/ 10field	0	0	0	0	0	3	2	0	3	2	3	1	0
1 cell/1field	0	0	0	0	0	0	3	0	1	3	1	0	1
>1cell/field	0	0	0	0	0	5	0	0	0	0	0	0	1
10cell/field	0	0	0	0	0	0	0	0	0	0	0	0	0
cell inflammatory foci:													
1cell/ 10field	3	3	4	4	5	4	3	5	4	5	0	4	0
1 cell/1field	0	0	0	0	0	0	2	0	1	0	4	1	0
>1cell/field	0	0	0	0	0	0	0	0	0	0	1	0	0
10cell/field	0	0	0	0	0	0	0	0	0	0	0	0	0
Mitosis:													
1cell/10field	2	0	0	0	1	1	1	1	1	0	2	4	0
1 cell/1field	0	0	0	0	0	1	2	0	1	4	0	0	0
>1cell/field	0	0	0	0	3	2	2	0	2	1	2	0	0
10cell/field	0	0	0	0	1	1	0	0	0	0	0	0	0
Greenish yellow pigmentation in Kupffer cells and hepatocytes													
1cell/10field	0	0	0	0	0	0	3	2	1	1	3	3	0
1cell/1field	0	0	0	0	0	5	2	2	0	4	1	2	0
Eosinophilic bodies and phagocytosis of red blood cells in hepatocytes:													
	0	0	0	0	0	0	1	0	0	2	0	0	3
Transparency													
1/3cells/field	1	1	1	0	3	3	1	3	3	2	1	3	0
1/2 cells/field	0	0	0	1	1	0	0	0	1	2	1	1	0
all cells/field	0	0	0	2	0	1	0	0	0	0	0	0	0
Hypertrophy:													
10%/10field	0	0	0	0	0	1	2	2	0	0	1	4	0
1/3 cells/field	0	0	0	1	3	3	1	1	2	3	1	1	3
1/2 cells/field	0	0	0	0	2	1	2	0	3	1	1	0	0
all cells/field	0	0	0	0	0	0	0	0	0	1	2	0	0

Dose/ endpoint	0				3000 ppm				5000 ppm				10 000 ppm
Week	**1**	**2**	**4**	**6**	**1**	**2**	**4**	**6**	**1**	**2**	**4**	**6**	**1**
Phagocytosis of red blood cells:													
10%/10field	2	0	0	0	1	1	1	1	1	0	2	4	0
1/3 cells/field	0	0	0	0	0	1	2	0	1	4	0	0	0
1/2 cells/field	0	0	0	0	3	2	2	0	2	1	2	0	0
all cells/field	0	0	0	0	1	1	0	0	0	0	0	0	0

Rec = recovery ; week 6 means 4 week treatment + 2 week recovery.

Conclusion:
Liver is the target organ in mice.
NOAEL < 3000 ppm (<600 mg/kg bw/d).
Material and methods:
20 male mice ICR strain /dose received in their diet, pyraflufen-ethyl (98.6% ; 4AM0021D) at 3000, 5000 or 10000 ppm for 1, 2 or 4 weeks. Five animals /group were sacrificed at day 0, 7, 14, 28 or 42 (this group is a recovery group).
The study is accepted.

B.5.8.1 Summary of further toxicological studies (Annex IIA 5.8)

4 week exposure to high doses of pyraflufen-ethyl (100 -2000 mg/kg bw/d) lead to accumulation of porphyrin in male rats (200 mg/kg bw/d) and mice (600 mg/kg bw/d) mainly in liver and kidney. These results clearly show that no porphyrin accumulation occurred in skin. According to the open literature, photosensitivity and related dermopathy are the prominent problems caused by deficiency of uroporphyrinogen decarboxylase, or uroporphyrinogen III synthase or ferrochelatase. However, photosensitivity is also characteristic of protoporphyrinogen oxidase deficiency, known as *Variegate porphyria* which is preferentially classified as hepatic porphyria, because biochemical expresssions are especially prominent in liver (Ellefson and Ford, 1996).
These studies confirm that rats, in general, were less susceptible to porphyriopathies than mice.The presently available data strongly suggest a species sensitivity.
No NOAEL was defined in mice. This point is adressed to the notifier as mice being the most sensitive species, it is necessary to define a NOAEL for porphyrin accumulation in liver before final ADI and AOEL can be proposed.
In rats, a NOAEL of 40 mg/kg bw/d is acceptable.

At 600 mg/kg bw/d, after 2 week exposure, single cell hepatocellular necrosis rarely accompanied with cell inflammation occurred was reported in mice. This effect appeared earlier at 1000 mg/kg bw/d and while recovery was complete at 600 mg/kg bw/d, some small liver necrosis was still present in 1 animal at 1000 mg/kg bw/d. Liver cell necrosis was followed by mitosis.
The hepatocellular necrosis, cell inflammatory foci, mitosis and hepatocellular hypertrophy recovered after 2 week pyraflufen-ethyl withdrawal but not completely.

A single oral dose of 5000 mg/kg bw is without significant effect on the mice liver weight, on the cytochrome P450 content and on the enzyme activities cytochrome P450 dependent. At 10000 mg/kg bw, decrease of CYP isoenzymes possibly reflecting a relative deficiency of heme for the responsible cytochromes isoforms occurred.
Oral administration of pyraflufen-ethyl for 28 days to mice at 1000 mg/kg bw/d induced a significant reduction of E.R.O.D., P.R.O.D., E.C.O.D, aniline hydroxylase and aminopyrine demethylase activities. NOAEL = 200 mg/kg bw/d is acceptable.

After 7 days oral administration of 1000 mg/kg bw/d pyraflufen-ethyl to mice, liver catalase activity was strongly reduced and associated with an increase in lipid peroxidation and beta-oxidation activity which may suggest proliferation of liver peroxisomes. At the top dose of 2 000 mg/kg bw/d, formation of 8-OH-dG was increased in liver DNA. NOAEL = 200 mg/kg bw/d is acceptable.
Peroxisome proliferators are well known rodent hepatocarcinogens. The relevance of hepatocarcinogenic effects of known peroxisome proliferators is often considered to be negligible for human hazard assessment.

Pyraflufen-ethyl feeding to mice produced, at high doses, a clear evidence of liver cell damage and death (necrosis) resulting from a cytotoxic effect resulting probably from the accumulation of porphyrins known to induce liver cell necrosis and inflammation followed by regenerative liver growth (Knasmûller et al., 1997). Increased mitotic activity is observed and it is assumed that the enhanced mitotic activity is of regenerative nature, serving to replace lost cells. This sustained induction of both adaptative and regenerative liver growth is associated with the formation of liver adenoma, reaching statistical significance at 5000 ppm in mice.
If there is a link between porphyria and hepatocellular adenoma formation is not clear.
Extrapolation from these experiments to man is difficult, however, certain human porphyrias are clearly associated with an increased risk for hepatocellular carcinoma.
The lowest NOAEL reported in these further toxicological studies is reported in the rat study, 40 mg/kg bw/d. At this dose, no significant increase in hepatic porphyrin was reported.

Table B.5.8.-1 : Summary of mechanistic studies of pyraflufen-ethyl :

Type of test	Compound and test substance purity	Results			References
		NOAEL (mg/kg bw /day)	LOAEL (mg/kg bw/day)	Critical endpoints	
mice, 28 day, oral	B.n°.4AM0021D 97.6 %	<600	600	ì porphyrin in tissues	Nakatani, 1998
rat, 28 day, oral	B.n°.4AM0021D 97.6 %	40	200	ì porphyrin in tissues	"
Mice, oral, 28 day	B.n°.4AM0021D 97.5 %	200	1000	liver weight ì;îlevel cyt.P450 and associated enzyme activities	Amanuma, 1996
Mice, oral, 7 day	94-T-0055 97.5%	200	1000	ì peroxisomal proliferation, ì lipid peroxides, ìcatalase activity	Inagaki, 1998
Mice, oral, 28 day	B.n°.4AM0021D 98.6 %	<600	600	ìALT, ìLiver weight; single cell necrosis, mitosis, pigments in Kupffer cells	Nakatani, 1994

B.5.9 Medical data (Annex IIA 5.9)

B.5.9.1 Report on medical surveillance on manufacturing plant personnel (Annex IIA 5.9.1)

Since 1988, pyraflufen- ethyl and its related compounds have been extensively investigated by research staff based at Nihon Nohyaku's research facilities at Osaka in Japan. Research has covered laboratory scale synthesis, preparation of the formulations, spray application under greenhouse and field conditions, toxicological studies and development of analytical methods. In addition kilogram scale synthesis of pyraflufen-ethyl has also been carried out at the Nihon Nohyaku's plant.

All workers at the research and production facilities are regularly screened for general health status by qualified personnel. During the research work on pyraflufen-ethyl or its related compounds, there have been no incidents or indications at all to be connected with any adverse effects caused by these chemicals.

B.5.9.2 Report on clinical cases and poisoning incidents (Annex IIA 5.9.2)

Pyraflufen-ethyl was not under commercial production at the time of this submission and there are not related cases of exposure or of poisoning incidents.

B.5.9.3 Observations on exposure of the general population and epidemiological studies (Annex IIA 5.9.3)

Pyraflufen-ethyl was not under commercial production at the time of this submission and there are therefore no epidemiological assessments or observation on experience of the general population.
Pyraflufen-ethyl affects the heme synthetic pathway and it is reported in the open literature that porphyrin accumulation may be consecutive to the exposure to enzyme inhibitors of the heme biosynthetic pathway. Pyraflufen-ethyl acts specifically on protoporphyrinogen oxidase and its inhibition leads to accumulation of PP IX. Protoporphyrinogen accumulates in the mitochondria and diffuse into the cytoplasm, where it spontaneously oxidizes to form PP IX. PP IX is an efficient photosensitizer and is capable of generating toxic species *in vivo* (Fingar et al., 1997). Accumulation of photoreactive by- products, the porphyrins, causes cutaneous photosensitivity and dermopathic manifestations and is also associated with neurological dysfunction and a propensity to acute neurovisceral crises. It is further reported that photosensitivity and related dermopathy are the prominent problems

caused by deficiency of uroporphyrinogen decarboxylase, or uroporphyrinogen III synthase or ferrochelatase. However, photosensitivity is also characteristic of protoporphyrinogen oxidase deficiency, known as *Variegate porphyria* which is preferentially classified as hepatic porphyria, because biochemical expresssions are especially prominent in liver (Ellefson and Ford, 1996).
Mild disturbances of the heme-forming system induced by toxic substances, often indicated by mild porphyrinuria, may be nonpathogenic but coincidental with pathogenesis inflicted on other systems by the toxins.

B.5.9.4 Clinical signs and symptoms of poisoning and details of clinical tests (Annex IIA 5.9.5)

The acute toxicity of pyraflufen-ethyl is extremely low. In the acute rat test, there were no clinical symptoms even at a dose of 5000 mg/kg bw.
Short-term oral exposure in the rat has revealed the development of reversible hematological changes and reversible effects on the liver and kidneys. Any effects would not be expected to occur at doses at which human exposure can occur. Realistic handling of pyraflufen-ethyl does not represent a significant risk to human.

From the open literature, photosensitive skin diseases, recurring abdominal colic, episodes of psychiatric distress, nausea and vomiting, fever, paresthesia, numbness, dysesthesia, hypertension, tachycardia and seizures are characteristics of porphyrias (Ellefson and Ford, 1996).
The diagnosis depends on fecal excretion of porphyrins, which is greatly increased in *variegate porphyria* and consists predominantly of protoporphyrin.
Porphyria are therefore, enzymatic defects which could be acquired after chronic exposure to chemicals interfering with human porphyrin biosynthesis (Rio et al., 1997)
There exist a lot of diagnostic laboratory tests including quantitation of porphyrins in blood, urine and feces and analysis of activities of enzymes of the heme-forming system. (Ellefson and Ford, Regul.Toxicol.Pharmacol.,24, S119-S125, 1996).
The definitive diagnostic tests for *variegate porphyria* is quantification of the protoporphyrinogen oxidase in hepatocytes, because of the occurrence of major expression in hepatocytes (Ellefson and Ford, 1996).

B.5.9.5 First aid measures - Therapeutic regimes (Annex IIA 5.9.5)

In case of contact with skin, remove contaminated clothing and wash affected area with soap and water.

If swallowed, wash out mouth with water, keep patient at rest and obtain immediate medical aid (show product label or the material safety data sheet if possible). Do not induce vomiting.

If inhaled, remove to fresh air and obtain medical aid if symptoms persist.

Inn case of contact with eyes, rinse immediately with water for at least 15 minutes and obtain medical aid at once.

From the open literature, in cases of unrecognized porphyria, acute attacks have been provoked or worsened by medications prescribed for relief from porphyric distress. Barbiturates, sulfonamide antibiotics, carbamazepine and diphenylhydantoin have been among the most used problematic medications. In preparation for surgery, induction of anesthesia with a barbiturate can induce catastrophe.

B.5.9.6.1 Expected effects and duration of poisoning as a function of the type, level and duration of exposure or ingestion (Annex IIA 5.9.6)

Any effects would not be expected to occur at doses at which human exposure can occur. It is concluded that there is sufficient evidence to be sure that the realistic handling of pyraflufen-ethyl does not represent a significant risk to human.

B.5.9.6.2 Expected effects and duration of poisoning as a function of varying time periods between exposure or ingestion and commencement of treatment (Annex IIA 5.9.6)

The acute toxicity of pyraflufen-ethyl is extremely low.

Any effects would not be expected to occur at doses at which human exposure can occur. It is concluded that there is sufficient evidence to be sure that the realistic handling of pyraflufen-ethyl does not represent a significant risk to human.

Most forms of porphyrias are inherited as Mendelian autosomal dominants, some types are recessive and others acquired through exposure to porphyrogenic drugs and chemicals. Compounds such as photobleaching herbicides, DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine) antineoplastic drugs, griseofulvin, hexachlorobenzene etc... may also inhibit heme synthesis *in vivo* and thereby cause porphyrin accumulation. Simultaneous or successive exposure to compounds affecting heme synthesis may be deleterious for people who carry a latent hereditary trait.

B.5.10 Summary of mammalian toxicology and proposed ADI, AOEL and drinking water limit (Annex IIA 5.10)

Metabolism :

After oral administration, pyraflufen-ethyl showed a rapid, dose-dependent and saturable absorption from the gastro-intestinal tract.
An absorption rate of 56% was extrapolated from urinary and biliary excretion (36.09% in bile + 19.66% in urine) for low dose. Increasing the dose, increased fecal excretion (± 90%) and reduced simultaneously urinary excretion to 3.6%. Absorption after oral high dose represents ± 20% of the dose.
Pyraflufen-ethyl has a limited distribution in organs and tissues, and 96 h after dosing, there were no tissues or organs exhibiting specifically retained radioactivity. At 3, 6 or 9 h postdose, the highest radioactivity was associated with the gastro-intestinal tract and the organs of metabolism and elimination, i.e. liver and kidney.
There was no evidence of accumulation.
Metabolism in rats as well as in plants, involves ester hydrolysis, O-dealkylation of the ether, N-demethylation on the pyrazole ring and further transformations of the phenoxyacetate into more polar metabolites. These metabolic pathways produce essentially 2 metabolites identified as E-1 and E-9 and other metabolites present at very low levels.
Excretion after oral administration was mainly fecal, representing ± 66% of a low dose, reaching 90% after high dose from which ± 78% was unchanged pyraflufen-ethyl. Urinary excretion represented 29-33% of the low dose and decreased to 2.5-5% after high dose. There was no evidence of accumulation.

Acute toxicity :

In rats and mice, acute oral toxicity was low and no mortality occurred after oral administration of 5000 mg/kg bw pyraflufen-ethyl. Moreover, no particular symptoms were recorded in rats. Male mice appeared more sensitive and some symptoms suggestive of an autonomic nervous sytem toxicity were observed.
Dermal toxicity was low but at the dose of 2000 mg/kg bw, male rats showed a significant reduced body weight at the 14th day.
A single oral dose of 5000 mg/kg bw is without significant effect on the mice liver weight, on the cytochrome P450 content and on the enzyme activities cytochrome P450 dependent. At 10000 mg/kg bw, decrease of CYP isoenzymes possibly reflecting a relative deficiency of heme for the responsible cytochromes isoforms occurred.

Pyraflufen-ethyl is not an eye and skin irritant, and is not a sensitizer.

Genotoxicity:

Genotoxicity of pyraflufen-ethyl was tested in a variety of different tests covering both, eukaryotes and prokaryotes *in vivo* and *in vitro*.
The aspect of mutagenicity is considered to be adequatly investigated.
While no point mutations were observed in bacterial cells, positive results were obtained in mouse lymphoma cells in the presence of metabolic activation. As the forward mutation study showed a slight positive evidence in the presence of metabolic activation, it was required from the notifier to confirm these results in a new study. A second test was conducted using lymphoma cells in which a clear negative response was observed.
Pyraflufen-ethyl was negative in the DNA repair test using the *Bacillus subtilis* rec assay as indicator, and did not cause DNA damage in rat liver *in vivo*. Pyraflufen-ethyl did not induce clastogenic or other damage *in vivo* in mice.
In conclusion, pyraflufen-ethyl is not genotoxic.

Short-term toxicity :

Oral administration of pyraflufen-ethyl to rats or mice for 4 weeks resulted in the expression of diminished availability of vital hemoproteins : males were more sensitive and showed reduced body weight, reduced erythrocyte characteristics, increased plasma bilirubin indicative of mild hemolytic anemia associated with a compensatory response from the bone marrow (in rats) and from the spleen (extramedullary haematopoieisis in rats; increased weight in mice). Liver weight increase was associated with increase in AP, ALT, AST, suggesting liver dysfunction and necrosis. (See supplementary studies).
Oral administration to rats of pyraflufen-ethyl for 13 weeks confirmed the erythrocytes and liver as targets of toxicity. Minor renal effects were also reported. The NOAEL in this study was 5000 ppm (455 mg/kg bw/d).

Mice seems to be the most sensitive species : at 523.7 mg/kg bw/day, anemia and liver toxicity was reported. In this study a NOAEL of 20 mg/kg bw /d was retained. This value was also the lowest NOAEL in short-term toxicity studies and therefore used for the calculation of the AOEL.
Dogs did not shown any toxic effect after 13 or 52 week administration.

Long-term toxicity studies :

In both long-term studies, target organs were blood, liver and kidney as also observed in the short-term toxicity studies.
In rats, chronic oral administration induced microcytic anemia. In kidneys, renal papilla were affected. Cytotoxicity observed in liver was probably consecutive to the oxidative stress induced by an increase in liver porphyrin which is characterized in rats, at 10000 ppm, by bile duct hyperplasia, electron-lucent vacuoles in hepatocytic mitochondria wtihout impairment of mitochondrial function and in mice, at 1000 ppm, by liver swelling, cytoplasmic vacuolation, pigment accumulation in Kupfer cells, foci of cellular alterations, liver hyperplasia (PCNA) and benign hepatocellular adenomas (at 5000 ppm). The NOAEL for proliferative activity of hepatocytes is 200 ppm (= 20 mg/kg bw/d). Porphyrin acccumulation in liver were not measured after long-term exposure.
Pyraflufen-ethyl was not carcinogenic in the rat study.
From both rat and mice studies, a NOAEL of 20 mg/kg bw/day is provisionally acceptable waiting for a NOAEL for porphyrin accumulation in liver in mice.
It is concluded that the increased hepatocellular adenomas in the high dose group of the mouse carcinogenicity study might be induced through the non genotoxic/cytotoxic mode of action of pyraflufen-ethyl.
From the open literature, two retrospective studies and one case-control study have suggested an association between primary liver cancer and acute hepatic porphyrias and according to the authors, acute hepatic porphyria shoulde be considered as a rare cause of hepatocellular carcinoma (Andant et al., 1998).

Reproductive toxicity:

In the two generation study, parental animals receiving pyraflufen-ethyl at the highest dose revealed a decreased body weight gain and pathological changes in liver and kidneys, and their offspring showed a decreased body weight gain during lactation period. However there were no abnormalities in the reproductive performance of parental animals and the reproductive index of offspring. Rats did not show any treatment-related changes in the teratology study.
Rabbits appeared to be strongly sensitive to the toxic effects of pyraflufen-ethyl. Due to the high mortality and insufficient number of pregnant rabbits at the 2 top doses, the study was not accepted by the RMS. This point is adressed to the notifier.

Supplementary studies :

Supplementary studies were performed in male mice or rats, after short-term exposure.
Feeding to male mice produced, at high doses, a clear evidence of liver cell damage and cell death (necrosis) consecutive to an oxidative stress and/or cytotoxicity resulting probably from the accumulation of porphyrins known to induce liver cell necrosis and inflammation followed by regenerative liver growth :

- Porphyrin accumulation, during a 4 week exposure period, was mainly observed in liver and kidneys of male rats (200 mg/kg bw/d) and mice (600 mg/kg bw/d). No porphyrin accumulation was observed in skin. This may be explained by the fact that photosensitivity and related dermopathy are the prominent problems caused by deficiency of uroporphyrinogen decarboxylase, or uroporphyrinogen III synthase or ferrochelatase. Photosensitivity is also characteristic of protoporphyrinogen oxidase deficiency, known as *Variegate porphyria* but this type of porphyria is preferentially classified as hepatic porphyria, because biochemical expressions are especially prominent in liver (Ellefson and Ford, 1996). The presently available data strongly suggest a species sensitivity.
No NOAEL was defined in the mice study for porphyrin accumulation. This point is adressed to the notifier as mice being the most sensitive species, it is necessary to define a NOAEL for this specific effect in liver before final ADI and AOEL can be proposed.
The lowest NOAEL reported in these further toxicological studies is reported in the rat study, at 40 mg/kg bw/d. At this dose, no significant increase in hepatic porphyrin was reported.
- At 1000 mg/kg bw/d a significant reduction of E.R.O.D., P.R.O.D., E.C.O.D, aniline hydroxylase and aminopyrine demethylase activities was observed. NOAEL = 200 mg/kg bw/d is acceptable.

- After 2 week exposure, single cell hepatocellular necrosis rarely accompanied with cell inflammation was reported in mice. While this effect recovered completely at 600 mg/kg bw/d after 2 week withdrawal, at 1000 mg/kg bw/d, some small liver necrosis were still present in 1 animal. It is assumed that the enhanced mitotic activity is of regenerative nature, serving to replace lost cells. The hepatocellular necrosis, cell inflammatory foci, mitosis and hepatocellular hypertrophy recovered after 2 week pyraflufen-ethyl withdrawal, but not completely. This sustained induction of both adaptative and regenerative liver growth is associated with the formation of liver adenoma, reaching statistical significance at 5000 ppm in mice after 2 year exposure. If there is a link between porphyria and hepatocellular adenoma formation is not clear.
- After 7 days oral administration of 1000 mg/kg bw/d pyraflufen-ethyl to mice, liver catalase activity was strongly reduced and lipid peroxidation and beta-oxidation activity were increased suggesting proliferation of liver peroxisomes. At the top dose of 2 000 mg/kg bw/d, formation of 8-OH-dG was increased in liver DNA. NOAEL = 200 mg/kg bw/d is acceptable.

Overall, extrapolation from these experiments to man is difficult, however, certain human porphyrias are clearly associated with an increased risk for hepatocellular carcinoma. Peroxisome proliferators are well known rodent hepatocarcinogens and the relevance of hepatocarcinogenic effects of known peroxisome proliferators is often considered to be negligible for human hazard assessment.

B.5.10.1 Acceptable daily intake (ADI)

A provisional ADI can be calculated from a NOAEL of 20 mg/kg bw/d (lowest NOAEL), identified in the 2 year rat and mice studies. Applying an assessment factor of 100 (10 for interspecies variation x 10 for intraspecies variation), the acceptable daily intake becomes :

ADI = 0.2 mg/kg bw/day

This value is a provisional proposal, waiting for futher information and NOAEL for porphyrin accumulation in mice liver.
An increase in benign hepatocellular adenoma was reported in the 78 week mouse study, at 540 mg/kg bw/day. Using an assessment factor of 100 is sufficient as the safety margin between the proposed ADI and the LOAEL is 2700.

B.5.10.2 Acceptable operator exposure (AOEL)

The AOEL short-term for man is calculated on the basis of an internal NOAEL from a sub-chronic animal experiment, taking into account the apparent degree of absorption, and applying an assessment factor, chosen in function of the critical effect observed in the animal experiments.

It appears from the animal experiment that a subchronic exposure to 20 mg/kg bw/d (lowest NOAEL, taken from the 90 day, mice study) of pyraflufen-ethyl will not result in any toxic effect. This value is a provisional proposal, waiting for futher information and NOAEL for porphyrin accumulation in mice liver. The oral absorption seems to be 56 %. Applying an assessment factor of 100 for extrapolation to man. The acceptable operator exposure level, expressed as an internal, systemic dose becomes:

AOEL systemic = 0.112 mg/kg bw/d

The notifier proposed a AOEL based on the 28-day oral rat study in which a NOEL of 230 mg/kg bw/day was obtained. Taking a factor of 25, this gives an AOEL = 9.2 mg/kg bw/d.

B.5.10.3 Drinking water limit

On the basis that exposure through drinking water should not account for more than 10% of the ADI, assuming an average consumption of 2 l of water per day and an average body weight of 70 kg, a limit of 0.7 mg/l is proposed.

$$\mathbf{MAC = \frac{ADI \times bw \times P}{C} = \frac{0.2 \times 70 \times 0.1}{2} = 0.7\ mg/l}$$

Milan SC

Milan SC is a suspension concentrate formulation containing the active ingredients pyraflufen-ethyl (9g/l) and bifenox (500g/l) which is used as a foliar application in post-emergence of cereals. Such formulation is designed for tractor mounted boom sprayers.

B.5.11 Acute toxicity including irritancy and skin sensitization of the preparations (Annex IIIA 7.1)

B.5.11.1 Acute oral toxicity (Annex IIIA 7.1.1)

- Milan SC, rat, 2006 mg/kg bw (Mercier, 1996a)

Findings:
Mortality : no mortality was observed.
Mean *bodyweight* was not affected.
Clinical signs : no abnormalities
Necropsy : no abnormalities.

Conclusions:
LD_{50} male > 2006 mg/kg bw
LD_{50} female > 2006 mg/kg bw

Guidelines :
Experimental protocol in compliance with method B.1 Annex V. Directive 92/69/EEC, or OECD 401(1981-1987)
GLP :
Yes
Material and Methods :
5 Albino rats (Sprague-Dawley)/sex received a single intragastric intubation of EXP31279 (B.n°. OP.951099 ; liquid) at 2006 mg/kg bw.

B.5.11.2 Acute percutaneous toxicity (Annex IIIA 7.1.2)

- Rat, dermal application, 2006 mg/kg bw, semi-occluded (Mercier, 1996b)

Findings:
No mortality, abnormal signs were observed in this study. At autopsy, no deviations from normal morphology were found.

Conclusion:
LD_{50} combined > 2006 mg/kg bw

Guidelines:
Experimental protocol in compliance with method B.3 Annex V of Directive 92/69/EEC.
GLP :
Yes
Material and Methods :
5 Rats (Ico: OFA.SD) were dermally exposed to EXP31279 (B.n°. OP.951099 ; liquid) undiluted at 2006 mg/kg bw under semi-occluded dressing for 24 h.

B.5.11.3 Acute inhalation toxicity to rats (Annex IIIA 7.1.3)

No data, not necessary.

B.5.11.4 Skin irritation (Annex IIIA 7.1.4)

- 3 Rabbit, 0.5 ml, semi-occluded patch 4 hours (Mercier, 1996c)

Findings:
Evaluation of the data according to the EU methodology, gave the following results:

< Score erythema > 24+48+72 h =0
< Score oedema > 24+48+72 h = 0

Conclusions :
Milan SC is not a skin irritant.

Guidelines :
Protocol in compliance with method B.6 of directive 92/69/EEC or OECD guideline 406(1981)
GLP :
Yes
Material and Methods :
Shaved skin of 3 male New Zealand white rabbits (NZW I.N.R.A. A9077) was exposed to 0.5 ml EXP31279 (B.n°. OP.951099 ; liquid) under semi-occluded dressing for 4 hours.

B.5.11.5 Eye Irritation (Annex IIIA 7.1.5)

- 3 Rabbits, 0.1 ml (Mercier, 1996d)

Findings:
< Score cornea opacity > 24+48+72h =0
< Score iris > 24+48+72 h =0
< Score redness > 24+48+/72h =0
< Score chemosis > 24+48+72 h=0

Conclusions :
Milan SC is not irritating to eyes.

Guidelines :
Protocol in compliance with method B.5 of Directive 92/69/EEC.
GLP :
Yes
Material and Methods :
0.1 ml EXP31279 (B.n°. OP.951099 ; liquid) was placed into the conjunctival sac of the eye of each of 3 males New Zealand white rabbits (NZW I.N.R.A. A 9077)

B.5.11.6 Skin sensitization (Annex IIIA 7.16)

- Guinea pigs, modified Buehler test (Mercier, 1996e)

Findings :
No mortality was observed during the study. Body weight changes in treated animals were not influenced by treatment. The repeated applications of Milan SC provoked signs of irritation during induction in the treated group. After challenge, no lesions of delayed hypersensitivity were observed.

Conclusion :
Milan SC is not a skin sensitizer.

Guidelines :
Protocol in compliance with method B.6, Annex V, Directive 96/54/EEC.Buelher test.

GLP :
Yes
Material and Methods :
During induction, 9 six hour topical occlusive applications were conducted with 0.5 ml EXP31279 (B.n°. OP.951099 ; liquid). Induction period is followed with 10 days rest.
Epidermal challenge : the topical occlusive application for 6 hours was performed at 0.5 ml per animal.
20 Hartley white strain guinea pigs/sex and 10 control group animals were used.

B.5.11.7 Supplementary studies for combinations of plant protection products (tests as at points 7.1.1 to 7.1.6) (Annex IIIA 7.1.7)

No data, not necessary.

B.5.12 Dermal absorption (Annex IIIA 7.3)

B.5.12.1 Dermal absorption, *in vivo* in the rat (Annex IIIA 7.3)

There is no indication that the estimated acceptable operator exposure level (AOEL) may be exceeded ; therefore, up to now, the dermal absorption study is not required, but it is probable that if a new formulation is proposed with higher concentration of pyraflufen-ethyl, a dermal absorption study should be necessary because in these conditions, the AOEL should be exceeded.

B.5.12.2 Comparative dermal absorption, *in vitro* using rat and human skin (Annex IIIA 7.3)

No data. Not necessary for this formulation.

B.5.13 Toxicological data on non active substances (Annex IIIA 7.4 and point 4 of the introduction)

The formulation contains emulsifiers and solvents:

B.5.14 Summary of toxicity of the formulation Milan SC

Table B.5.14-1 : Summary of toxicity of Milan SC

Type of test Test species	**Test substance**	**Results**	**Classification**	**References**
Acute, oral, rat	EXP31279 B.n°. OP.951099 ; liquid	LD_{50} _,_ > 2006 mg/kg bw	-	**Mercier, 1996a**
Dermal, rat	EXP31279 B.n°. OP.951099 ; liquid	LD_{50} combined > 2006 mg/kg bw	-	**Mercier, 1996b**
Rabbit, skin irritation	EXP31279 B.n°. OP.951099 ; liquid	not irritant	-	**Mercier, 1996c**
Rabbit, eye irritation	EXP31279 B.n°. OP.951099 ; liquid	not irritating to eyes	-	**Mercier, 1996d**
Guinea pig, modified Buelher test	EXP31279 B.n°. OP.951099 ; liquid	not sensitizer	-	**Mercier, 1996e**

B.5.15 Exposure data (Annex IIIA 7.2)

B.5.15.1 Estimation of operator exposure (Annex IIIA 7.2.1.1)

The formulation Milan SC (pyraflufen-ethyl (9g/l) and bifenox (500g/l)) is designed for ground spray application (tractor mounted boom with hydraulic nozzles). Use rates are 1.5l/ha for late applications on developped weeds. In case of earlier applications, a reduced dose rate of 1.33l/ha can be applied. Tha maximum dose rate of 1.5l/ha correspond to 13.5 g pyraflufen-ethyl and 750 g active ingredient bifenox /ha. It is recommended to apply a preparation with 150 to 400 l water/ha.
Model calculations were made on the basis of UK-POEM and German model.

Applications parameters :
Table B.5.15.1-1 : Application parameters

Application technique	**Treatment of**	**Max. application rate (kg a.s./ha)**	**Water vol. (L/ha)**	**Highest spray cc (g a.s./hl)**	**Treatment area (ha)**
tractor mounted boom with hydraulic nozzles	foliar application - cereals	0.0135	150-400	68	50(UK) 20(G)

The following parameters were taken into account for exposure estimates :

	UK POEM	GERMAN MODEL
Use rate:	13.5 g a.s./ha	13.5 g a.s./ha
Number ha treated/day:	50	20
Application equipment:	tractor mounted with cab	field crop tractor mounted
Body weight:	60 kg	70 kg
AOELsytemic:	0.112 mg/kg bw/day	Itol = 7
AOEL dermal:	0.112 mg/kg bw/day	Dtol = 7 (100%) = 70 (10%)
Dermal absorption:	100% and 10%	100% and 10%

Protective equipment :
Calculations were made for scenarios with and without protective equipment:
- gloves worn during mixing/loading (UK model)
- gloves worn during mixing/loading and spraying, protective clothing, and sturdy footwear (German model).

Expected operator exposures :
Table B.5.15.1-2 : Estimated operator exposure (mg/person/day) according to the UK POEM model

Type of protection/dermal absorption	**Dermal absorbed dose (mg/person/day)**		**Inhalation exposure (mg/person/day)**	**Total absorbed dose (mg/ person/day)**
	Mix/load	**Spray**	**Spray**	
Type of protection : none				
dermal absorption : 100%	3.4	2.805	0.004	6.184
dermal absorption : 10%	0.3	0.280	0.004	0.622
Type of protection : gloves				
dermal absorption :				

100%	0.17	2.805	0.004	2.977
dermal absorption : 10%	0.02	0.280	0.004	0.301

Table B.5.15.1-3 : Estimated external operator exposure (mg/person/day) according to the German model

Type of protection/ dermal absorption	**Dermal exposure (mg/person/day)**			**Inhalation exposure (mg/person/day)**			**Total external exposure (mg/person /day)**
	Mix/ load	**Spray**	**Total**	**Mix/ load**	**Spray**	**Total**	
Type of protection : none							
100%or 10%	0.648	0.550	1.198	0.000162	0.00027	0.000432	1.199
Type of protection : gloves, standard protective garment, sturdy footwear							
100 or 10%	0.648	0.1404	0.788	0.000162	0.00027	0.000432	0.1473

Comparision of estimated and tolerable exposure :

Table B.5.15.1-4 : Exposure as a proportion of AOEL -UK model

	Total systemic exposure - 60 kg person (mg/kg bw/day)				**% of AOEL**			
	no PPE worn		**PPE worn**		**no PPE worn**		**PPE worn**	
Dermal absorption	**10%**	**100%**	**10%**	**100%**	**10%**	**100%**	**10%**	**100%**
	0.01	0.103	0.005	0.050	10	103	5	50

Table B.5.15.1-4 : Total degree of exposure E - German model

Total exposure - 70 kg person (mg/kg bw/day)		**E**			
no PPE worn	**PPE worn**	**no PPE worn**		**PPE worn**	
		Dtol=7	Dtol=70	Dtol=7	Dtol=70
1.1992	0.1473	0.1714	0.01718	0.0210	0.00214

Conclusions :
Exposure calculations performed according to German and UK models give results rather different in terms of exposure. Nevertheless, the following conclusions can be taken :
The operator exposure (% AOEL, degree of exposure E) with or without protective equipment is acceptable.This evaluation is however based on a provisional AOEL.

B.5.15.2 Measurement of operator exposure (Annex IIIA 7.2.1.2)

No data. Not necessary.

B.5.15.3 Estimation of bystander exposure (Annex IIIA 7.2.2)

It can be assumed that bystanders may be present during the field use of Milan SC and can therefore be considered to be exposed mainly by the airborne route. If exposure of a bystander compared with an operator is proportional to the airborne material it is likely that a bystander, outside the treatment area will not be exposed to a dose greater than the AOEL. (Inhalation exposure of the operator during spraying is 0.00405 mg/day which represents 3.6% of the AOEL).

B.5.15.4 Estimation of worker exposure (Annex IIIA 7.2.3.1)

Milan SC is applied on cereals crops. The product will be quickly absorbed into the top surface soil and will therefore not be readily transferred to workers. Also it is unnecessary to re-enter the field for further work following treatment. As there is no risk for the applicator, there are no risk during re-entry in the field.

B.5.15.5 Measurement of worker exposure (Annex IIIA 7.2.3.2)

No data. Not necessary.

B.5.15.4 Estimation of worker exposure (Annex IIIA 7.2.3.1)

No data. Not necessary.

B.5.15.5 Measurement of worker exposure (Annex IIIA 7.2.3.2)

No data. Not necessary.

B.5.16 References relied on

Toxicology and metabolism of the active substance (Annex IIA 5)

Author(s)	Year	Annex IIA Point Title Company, Report No.	GLP GEP Y/N	Published or not Y/N	Owner
Andant, Ch., and Puy, H.	1998	IIA 5.2, IIA 5.5Acute hepatic porphyrias and primary liver cancer. Reference: New England J. Medicine, 338 (25), 1853-1854, 1998	N	Y	-
Andersson, C., Bjersing, L. and Lithner, F.	1996	IIA 5.5The epidemiology of hepatocellular carcinoma in patients with acute intermittent porphyria. J. Inter. Med., 240, 1996, 195-201.	N	Y	-
Amanuma, T.	1995a	IIA, 5.2.1/01 Acute oral toxicity study of ET-751 technical in rat Nihon Nohyaku, Report No.: T-5019	Y	N	NN
Amanuma, T.	1995b	IIA, 5.2.1/02 Acute oral toxicity study of ET-751 technical in mice Nihon Nohyaku, Report No.: T-5017	Y	N	NN
Amanuma, T.	1995c	IIA, 5.2.2 Acute dermal toxicity study of ET-751 technical in rat Nihon Nohyaku, Report No.: T-5018	Y	N	NN
Amanuma, T.	1996	IIA, 5.8.2/01 Effect of ET-751 on hepatic drug metabolizing enzymes in mice Nihon Nohyaku, Report No.: T-5062	N	N	NN
Bloomer, JR.	1997	IIA 5.2.1Hepatic protoporphyrin metabolism in patients with advanced protoporphyric liver disease. Reference : Yale J. Biol. Med, 70(4)323-330, 1997.	N	Y	-
Bouvier, G.	1999	IIA 5 ET-751 : Absence of genotoxic hazard, and no requirement of further test for EU registration. Rhône-Poulenc Agro, Sophia Antipolis, France. 31 1999	N	N	RPA
Broadmeadow, A.	1994a	IIA, 5.3.1/01 ET-751: Preliminary toxicity study by dietary administration to CD rats for four weeks Nihon Nohyaku, Report No.: T-5001	Y	N	NN
Broadmeadow, A.	1994b	IIA, 5.3.2/01 ET-751: Toxicity study by dietary administration to CD rats for 13 weeks followed by an 8 week reversibility period Nihon Nohyaku, Report No.: T-5006	Y	N	NN
Broadmeadow, A.	1994c	IIA, 5.3.2/02 ET-751: Toxicity study by dietary administration to CD rats for 13 weeks followed by an 8 week reversibility period -First supplement to Pharmaco LSR Report No : 93/NHH058/1093, Electron microscopy report- Nihon Nohyaku, Report No.: T-5007	Y	N	NN
Broadmeadow, A.	1996a	IIA, 5.3.2/03	Y	N	NN

Author(s)	Year	Annex IIA Point Title Company, Report No.	GLP GEP Y/N	Published or not Y/N	Owner
		ET-751: Toxicity study by oral (capsule) administration to beagle dogs for 13 weeks Nihon Nohyaku, Report No.: T-5038			
Broadmeadow, A.	1996b	IIA, 5.3.2/04 ET-751: Toxicity study by oral (capsule) administration to beagle dogs for 52 weeks Nihon Nohyaku, Report No.: T-5061	Y	N	NN
Burns, L. M.	1995	IIA, 5.6.2/01 ET-751: Teratology study following oral (gavage) administration in the rat Nihon Nohyaku, Report No.: T-5021	Y	N	NN
Burns, L. M.	1996	IIA, 5.6.2/02 ET-751: Teratology study in the rabbit Nihon Nohyaku, Report No.: T-5034	Y	N	NN
Butterworth, B.E., Conolly, R.B. and Morgan, K.T.	1995	IIA 5 A strategy for establishing mode of action chemical carcinogens as a guide for approaches to risk assessments. 12 April 1995. Cancer Letters, 93, 129-146	N	Y	-
Cracknell, S.	1995	IIA, 5.2.3 ET-751: acute inhalation toxicity study in the rat Nihon Nohyaku, Report No.: T-5012	Y	N	NN
Dance, C. A.	1994	IIA, 5.4.1/02 ET-751: an in vitro test for induction of chromosome damage: cytogenetic study in cultured human peripheral lymphocytes Nihon Nohyaku, Report No.: T-5008	Y	N	NN
Edwards, C. N.	1994	IIA, 5.4.2 ET-751: mouse micronucleus test Nihon Nohyaku, Report No.: T-5004	Y	N	NN
Ellefson, R.D. and Ford, R.	1996	IIA 5.9.5The polyphyrias : characteristics and laboratory tests. Reference: Regul. Toxicol.Pharmacol., 1996, 24, S119-S125.	N	Y	-
Fingar, VH., Wieman, TJ., McMahon, KS., Halling, BP., Yuhas, DA, Winkelman, JW.	1997	IIA 5.9.3Phtotodynamic therapy using a protoporphyrinogen oxidase inhibitor. Reference: Cancer Res., 57(20),4551-4556, 1997.	N	Y	-
Fujii, S.	1996	IIA, 5.6.1 ET-751: Two-generation reproduction study in rats Nihon Nohyaku, Report No.: T-5026	Y	N	NN
Inagaki, K.	1994	IIA, 5.4.1/04 ET-751: DNA repair test (rec-assay) with *Bacillus subtilis* Nihon Nohyaku, Report No.: T-5011	Y	N	NN
Inagaki, K.	1998	IIA 5.8 Effect of pyraflufen-ethyl dietary adminstration on lipid	Y	N	NN

Author(s)	Year	Annex IIA Point Title Company, Report No.	GLP GEP Y/N	Published or not Y/N	Owner
		peroxidation, β-oxidative activity, catalase activity and 8-hydroxydeoxyguanosine production in mouse liver. Research Centre, Nihon Nohyaku Co., Ltd., Japan Report N° LSRC-T98-051A (Draft in English). July 1998. 7 pp.			
Kawamura, S., Yoshioka, T., Kato, T., Matsuo, M., Yasuda, M.	1996	IIA 5.2Histological changes in rat embryonic blood cells as a possible mechanism for ventricular septal defects produced by an N-phenylimide herbicide. Reference: Teratology, 54(5), 237-244, 1996.	N	Y	-
Knasmuller, S., Parzefall, W.,Helma Ch., Kassie, F., Ecker, S., and Schulte-Hermann, R.	1997	IIA 5 and IIA 5.9.3Reference: Crit. Rev. Toxicol., 27(5), 495-537, 1997	N	Y	-
Komatsu, M.	1995a	IIA, 5.2.4 Primary dermal irritation study of ET-751 technical in rabbit Nihon Nohyaku, Report No.: T-5015	Y	N	NN
Komatsu, M.	1995b	IIA, 5.2.5 Primary eye irritation study of ET-751 technical in rabbit Nihon Nohyaku, Report No.: T-5014	Y	N	NN
Krijt, J., Holsteijn, I., Hassing, I., Vokurka, M., and Blaauboer, B.	1993	IIA 5.2Effect of diphenyl ether herbicides and oxadiazinon on porphyrin biosynthesis in mouse liver, rat primary hepacyte culture and HepG2 cells. Reference: Arch. Toxicol., 67, 255-261, 1993	N	Y	-
Kuwahara, M.	1996	IIA, 5.5/02 ET-751: 78-week oral oncogenicity study in mice Nihon Nohyaku, Report No.: T-5060	Y	N	NN
Kuwahara, M.	1997	IIA, 5.8.2/02 ET-751: 78-week oral oncogenicity study in mice - Additional study of effect on proliferative activity of hepatic cells Nihon Nohyaku, Report No.: T-5066	Y	N	NN
Lindal, S., Torbergsen, T., Aasly, J., Mellgren, SI., and Monstad, P.	1992	IIA 5.3.2Mitochondrial diseases and myopathies : a series of muscle biopsy specimens with ultrastructural changes in the mitochondria. Reference: Ultrastruct. Pathol., 16 (3), 263-275, 1992	N	Y	-
Lloyd, J. M.	1994	IIA, 5.4.1/03 ET-751: L5178Y TK+/- mouse lymphoma mutation assay using the methodology recommended by the O.E.C.D. (1984) Nihon Nohyaku, Report No.: T-5005	Y	N	NN

Author(s)	Year	Annex IIA Point Title Company, Report No.	GLP GEP Y/N	Published or not Y/N	Owner
Logan , GM., Weimer, MK., Ellefson, M., Pierach, CA., Bloomer, JR.	1991	IIA 5.2.1Bile porphyrin analysis in the evaluation of variegate porphyria. Reference: N. Engl.J.Med, 324 (20), 1408-1411, 1991	N	Y	-
May, K.	1994	IIA, 5.4.1/01 ET-751: Assessment of mutagenic potential in amino-acid auxotrophs of *Salmonella typhimurium* and *Escherichia coli* (the Ames test) Nihon Nohyaku, Report No.: T-5003	Y	N	NN
Meyer,U.A, Schuurmans, M.M. and Lindberg, RL.	1998	IIA 5.2.1Acute porphyrias : pathogenesis of neurological manifestations. Reference: Semin. Liver Dis. 18, 1998, 43-52.	N	Y	
Meyer, UA., Schuurmans, MM., and Lindberg, RL.	1998	IIA 5.3 Acute porphyrias : pathogenesis of neurological manifestations. Reference: Semin. Liver Dis., 18(1), 43-52, 1998	N	Y	-
Moore, MR.	1993	IIA 5.2.1Biochemistry of porphyria. Reference: Int. J. Biochem., 25(10), 1353-1368, 1993.	N	Y	-
Motoba, K.	1996a	IIA, 5.1/01 Absorption, distribution, metabolism and excretion of [pyrazole-5-^{14}C] ET-751 following a single oral administration to male and female rats Nihon Nohyaku, Report No.: T-5039	Y	N	NN
Motoba, K.	1996b	IIA, 5.1/02 Metabolism and excretion of [phenyl-U-^{14}C] ET-751 following a single oral administration to male and female rats Nihon Nohyaku, Report No.: T-5059	Y	N	NN
Motoba, K.	1996c	IIA, 5.1/03 Absorption, distribution, metabolism and excretion of a single oral dosing of [pyrazole-5-^{14}C] ET-751 following repetitive oral dosing of non-radiolabeled test substance to rats Nihon Nohyaku, Report No.: T-5046	Y	N	NN
Motoba, K.	1996d	IIA, 5.1/04 Metabolism and excretion of [pyrazole-5-^{14}C] ET-751 into bile following a single oral administration to rats Nihon Nohyaku, Report No.: T-5045	Y	N	NN
Nakatani, M.	1998	IIA 5.8 Studies on the porphyrin accumulation in mice and rats by pyraflufen-ethyl administration. Research Centre, Nihon Nohyaku Co., Ltd., Japan (Draft in English). Report code T-5095. July 1998. 11 pp.	Y	N	NN
Nakatani, M.	1998	IIA 5.8 Investigation of liver injury caused by dietary	Y	N	NN

Author(s)	Year	Annex IIA Point Title Company, Report No.	GLP GEP Y/N	Published or not Y/N	Owner
		administration of pyraflufen-ethyl in mice. Research Centre, Nihon Nohyaku Co., Ltd., Japan (Draft in English) Report n° LSRC-T98-0046. July 1998. 22 pp.			
Omara, F., Blakley, B. and Wanjala, L.	1993	IIA 5.3.2Hepatotoxicity associated with dietary iron overload in mice. Reference: Human & Experimental toxicol., 12 , 463-467, 1993	N	Y	--
Patel, S.	1996	IIA, 5.5/01 ET-751: Combined oncogenicity and toxicity study by dietary administration to CD rats for 104 weeks Nihon Nohyaku, Report No.: T-5064	Y	N	NN
Rees, P. B.	1995	IIA, 5.2.6 ET-751: Delayed contact hypersensitivity study in the guinea-pig Nihon Nohyaku, Report No.: T-5013	Y	N	NN
Rio B., D.Parent-Massin, S. Lautraite and H. Hoellinger.	1997	IIA 5.2Effects of a diphenyl-ether herbicide, oxyflurfen, on human BFU-E/CFU-E development and hemoglobin synthesis Reference: Human and Exp.Toxicol. 16, 1997, 115-122.	N	Y	-
Salonpaa, P., Kottari, S., Pelkonen, O. And Raunio, H.	1997	IIA 5.2.1Regulation of CYP 2A5 induction by porphyrogenic agents in mouse primary hepatocytes. Reference: Naunyn Schiedebergs Arch. Parmacol., 355, 8-13, 1997	N	Y	-
Tanaka, N.	1998	II A 5.4.1 Report on mutation Assay of ET-751 Using L5178Y Mouse Lymphoma Cells. Hatano Research Institute,Food and Drugs Safety Centre, Japan. HRI Report N° 10-426. Revised August 18, 1998.	Y	N	NN
Takahashi, K.	1994	IIA, 5.3.1/02 ET-751: 78-week oral oncogenicity study in mice / 28-day dose range finding study Nihon Nohyaku, Report No.: T-5002	Y	N	NN
Ye, Q., and Bodell, W.,	1996	IIA 5Production of 8-hydroxy-2'-deoxyguanosine in DNA by microsomal activation of tamoxifen and 4-hydroxytamoxifen. Reference: Carcinogenesis, 17, 1747-1750, 1996	N	Y	-

A.1.5.2a Toxicology and metabolism of the formulation MILAN (Annex III A 7)

Author(s)	Year	Annex IIA Point Title Company, Report No.	GLP GEP Y/N	Published or not Y/N	Owner
Mercier, O.	1996a	Annex IIIA, 7.1.1 EXP31279A - Single dose toxicity study by the oral route in the rat (Limit test) Report n°: 609316, 20 December 1996 Pharmakon Europe, L'Arbresle, France	Y	N	RPA
Mercier, O.	1996b	Annex IIIA, 7.1.2 EXP31279A - Single dose toxicity study by the cutaneous route in the rat (Limit test) Report n°: 609317, 20 December 1996 Pharmakon Europe, L'Arbresle, France	Y	N	RPA
Mercier, O.	1996c	Annex IIIA, 7.1.4 EXP31279A - Primary cutaneous irritation and corrosivity test in the rabbit (P.C.I.C.) - 3 rabbits Report n°: 609318, 20 December 1996 Pharmakon Europe, L'Arbresle, France	Y	N	RPA
Mercier, O.	1996d	Annex IIIA, 7.1.5 EXP31279A - Ocular irritation and reversability test in the rabbit (O.I.R.) - 3 rabbits Report n°: 609319, 20 December 1996 Pharmakon Europe, L'Arbresle, France	Y	N	RPA
Mercier, O.	1996e	Annex IIIA, 7.1.6 EXP31279A - Sensitization potential in the guinea pig - Modified Buehler Test (9 induction applications) Report n°: 609320, 23 December 1996 Pharmakon Europe, L'Arbresle, France	Y	N	RPA

ANNEX B

Pyraflufen-ethyl

Appendix C: Estimation of the Operator Exposure

UK -POEM
Vehicle mounted with cab, hydraulic nozzles. No gloves.

PRODUCT DATA	
Product	Milan SC
Active substance	pyraflufen-ethyl
Concentration	9 mg/ml
Formulation type	SC
Maximum in-use a.s. concentration	0.068 mg/ml

EXPOSURE DURING MIXING AND LOADING	
Container size	10 l
Hand contamination/operation	0.05 ml
Application dose	1.5 l product/ha
Work rate	50 ha/day
Number of operations	8 /day
Hand contamination	0.4 g/day
Protective clothing	none
Transmission to the skin	100%
Dermal exposure to formulation	0.4 ml/day

EXPOSURE DURING SPRAY APPLICATION
vehicle mounted with cab, hydraulic nozzles

Application volume	200 spray/ha		
Volume of surface contamination	10 ml/h		
Distribution	Hands	Trunk	Legs
	65	10	25
Clothing	none	coverall	coverall
Penetration	100	5	15%
Dermal exposure	6.5	0.05	0.375 ml/h
Duration of exposure	6 h		
Total dermal exposure to spray	41.55 ml/day		

ABSORBED DOSE

	Mixing/loading	Application
Dermal exposure	0.4 ml/day	41.55 ml/day
Concentration of a.s.	9 mg/ml	0.068 mg/ml
Dermal exposure to a.s.	3.4 mg/day	2.804 mg/day
Percent absorbed	100 %	100 %
Absorbed dose	3.4 mg/day	2.805 mg/day

INHALATION EXPOSURE DURING SPRAYING	
Inhalation exposure	0.01 ml/h
Duation of exposure	6 h
Concentration of a.s.	0.068 mg/ml
Inhalation exposure to a.s.	0.00405 mg/day
Percent absorbed	100%
Absorbed dose	0.004 mg/day

PREDICTED EXPOSURE	
Total absorbed dose	6.184 mg/day
Operator body weight	60 kg

Operator exposure	0.103 mg/kg bw/day	103 % AOEL

UK -POEM
Vehicle mounted with cab, hydraulic nozzles. No gloves.

PRODUCT DATA	
Product	Milan SC
Active substance	pyraflufen-ethyl
Concentration	9 mg/ml
Formulation type	SC
Maximum in-use a.s. concentration	0.068 mg/ml

EXPOSURE DURING MIXING AND LOADING	
Container size	10 l
Hand contamination/operation	0.05 ml
Application dose	1.5 l product/ha
Work rate	50 ha/day
Number of operations	8 /day
Hand contamination	0.4 g/day
Protective clothing	none
Transmission to the skin	100%
Dermal exposure to formulation	0.4 ml/day

EXPOSURE DURING SPRAY APPLICATION
vehicle mounted with cab, hydraulic nozzles

Application volume	200 spray/ha		
Volume of surface contamination	10 ml/h		
Distribution	Hands	Trunk	Legs
	65	10	25
Clothing	none	coverall	coverall
Penetration	100	5	15%
Dermal exposure	6.5	0.05	0.375 ml/h
Duration of exposure	6 h		
Total dermal exposure to spray	41.55 ml/day		

ABSORBED DOSE

	Mixing/loading	Application
Dermal exposure	0.4 ml/day	41.55 ml/day
Concentration of a.s.	9 mg/ml	0.068 mg/ml
Dermal exposure to a.s.	3.4 mg/day	2.804 mg/day
Percent absorbed	10 %	10 %
Absorbed dose	0.34 mg/day	0.2805 mg/day

INHALATION EXPOSURE DURING SPRAYING	
Inhalation exposure	0.01 ml/h
Duation of exposure	6 h
Concentration of a.s.	0.068 mg/ml
Inhalation exposure to a.s.	0.00405 mg/day
Percent absorbed	100%
Absorbed dose	0.004 mg/day

PREDICTED EXPOSURE

Total absorbed dose	0.6184 mg/day	
Operator body weight	60 kg	
Operator exposure	0.0103 mg/kg bw/day	10 % AOEL

UK -POEM
Vehicle mounted with cab, hydraulic nozzles.Gloves.

PRODUCT DATA	
Product	Milan SC
Active substance	pyraflufen-ethyl
Concentration	9 mg/ml
Formulation type	SC
Maximum in-use a.s. concentration	0.068 mg/ml

EXPOSURE DURING MIXING AND LOADING	
Container size	10 l
Hand contamination/operation	0.05 ml
Application dose	1.5 l product/ha
Work rate	50 ha/day
Number of operations	8 /day
Hand contamination	0.4 g/day
Protective clothing	gloves
Transmission to the skin	5%
Dermal exposure to formulation	0.02 ml/day

EXPOSURE DURING SPRAY APPLICATION
vehicle mounted with cab, hydraulic nozzles

Application volume	200 spray/ha		
Volume of surface contamination	10 ml/h		
Distribution	Hands	Trunk	Legs
	65	10	25
Clothing	none	coverall	coverall
Penetration	100	5	15%
Dermal exposure	6.5	0.05	0.375 ml/h
Duration of exposure	6 h		
Total dermal exposure to spray	41.55 ml/day		

ABSORBED DOSE

	Mixing/loading	Application
Dermal exposure	0.02 ml/day	41.55 ml/day
Concentration of a.s.	9 mg/ml	0.068 mg/ml
Dermal exposure to a.s.	0.17 mg/day	2.804 mg/day
Percent absorbed	100 %	100 %
Absorbed dose	0.17 mg/day	2.805 mg/day

INHALATION EXPOSURE DURING SPRAYING	
Inhalation exposure	0.01 ml/h
Duation of exposure	6 h
Concentration of a.s.	0.068 mg/ml
Inhalation exposure to a.s.	0.00405 mg/day
Percent absorbed	100%
Absorbed dose	0.004 mg/day

PREDICTED EXPOSURE		
Total absorbed dose	2.977 mg/day	
Operator body weight	60 kg	
Operator exposure	0.05 mg/kg bw/day	50 % AOEL

UK -POEM
Vehicle mounted with cab, hydraulic nozzles. Gloves.

PRODUCT DATA	
Product	Milan SC
Active substance	pyraflufen-ethyl
Concentration	9 mg/ml
Formulation type	SC
Maximum in-use a.s. concentration	0.068 mg/ml
EXPOSURE DURING MIXING AND LOADING	
Container size	10 l
Hand contamination/operation	0.05 ml
Application dose	1.5 l product/ha
Work rate	50 ha/day
Number of operations	8 /day
Hand contamination	0.4 g/day
Protective clothing	gloves
Transmission to the skin	5%
Dermal exposure to formulation	0.02 ml/day

EXPOSURE DURING SPRAY APPLICATION
vehicle mounted with cab, hydraulic nozzles

Application volume	200 spray/ha		
Volume of surface contamination	10 ml/h		
Distribution	Hands	Trunk	Legs
	65	10	25
Clothing	none	coverall	coverall
Penetration	100	5	15%
Dermal exposure	6.5	0.05	0.375 ml/h
Duration of exposure	6 h		
Total dermal exposure to spray	41.55 ml/day		

ABSORBED DOSE

	Mixing/loading	Application
Dermal exposure	0.02 ml/day	41.55 ml/day
Concentration of a.s.	9 mg/ml	0.068 mg/ml
Dermal exposure to a.s.	0.17 mg/day	2.804 mg/day
Percent absorbed	10 %	10 %
Absorbed dose	0.02 mg/day	0.2805 mg/day

INHALATION EXPOSURE DURING SPRAYING	
Inhalation exposure	0.01 ml/h
Duation of exposure	6 h
Concentration of a.s.	0.068 mg/ml
Inhalation exposure to a.s.	0.00405 mg/day
Percent absorbed	100%

Absorbed dose	0.004 mg/day	
PREDICTED EXPOSURE		
Total absorbed dose	0.301 mg/day	
Operator body weight	60 kg	
Operator exposure	0.005 mg/kg bw/day	5 % AOEL

German model for the determination of operator exposure				
1. Field crop tractor mounted				
PRODUCT DATA				
Product	Milan SC			
Active ingredient	pyraflufen-ethyl			
Concentration of the product (g/kg)	9			
Formulation type	SC			
Application technique	field crop tractor mounted			
Use rate (kg a.s./ha)	0.0135			
Area treated per day(ha)	20			
Absorption factor (%)	100			
AOEL CALCULATION	AOEL oral	AOELskin	AOELskin	AOELinhalation
NOAEL(mammal)(mg/kgbw/d)	20	20		
Body weight (kg)	70	70		
Safety factor	200	200		
AOEL(human)(mg/kg bw/d)	0.1	1.0		
	Otol	Dtol	Dtol	Itol
Tolerable exposure(human)(mg/person/d)	7	70	7	5.25
SPECIFIC VALUES OF EXPOSURE FOR THE TYPE OF APPLICATION				
D*M(H)(mg/person*kg a.s.)	2.4		x 0.01	
D*A(H)(mg/person*kg a.s.)	0.38			
D*A(B)(mg/person*kg a.s.)	1.6		x 0.05	
D*A(C)(mg/person*kg a.s.)	0.06			
I*M(mg/person*a.s.)	0.0006			
I*A(mg/person*kg a.s.)	0.0.001			
ESTIMATION OF OPERATOR EXPOSURE				
	No protection		PPE	
Mixing				
DM(H)	0.648		0.00648	
IM	0.000162		0.000162	
Application				
DA(H)	0.1026		0.1026	
DA(B)	0.432		0.0216	
DA(C)	0.0162		0.0162	
IA	0.00027		0.00027	
RISK ASSESSMENT				
DM(H)/Dtol	0.00925		9.26 E-05	
DA(H)/Dtol	0.001466		0.001466	
DA(B)/Dtol	0.00617		0.000309	

DA(C)/Dtol	0.000231	0.000231
IM/Itol	2.31 E-05	2.31 E-05
IA/Itol	3.86 E-05	3.86 E-0.5
Degree of exposure (E)	**0.01718**	**0.002137**

German model for the determination of operator exposure				
1. Field crop tractor mounted				
PRODUCT DATA				
Product	Milan SC			
Active ingredient	pyraflufen-ethyl			
Concentration of the product (g/kg)	9			
Formulation type	SC			
Application technique	field crop tractor mounted			
Use rate (kg a.s./ha)	0.0135			
Area treated per day (ha)	20			
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AOEL CALCULATION	AOEL oral	AOELskin	AOELskin	AOELinhalation
NOAEL(mammal)(mg/kgbw/d)	20	20		
Body weight (kg)	70	70		
Safety factor	200	200		
AOEL(human)(mg/kg bw/d)	0.1	1.0		
	Otol	Dtol	Dtol	Itol
Tolerable exposure (human) (mg/person/d)	7	7	7	5.25
SPECIFIC VALUES OF EXPOSURE FOR THE TYPE OF APPLICATION				
D*M(H) (mg/person*kg a.s.)	2.4		x 0.01	
D*A(H) (mg/person*kg a.s.)	0.38			
D*A(B)(mg/person*kg a.s.)	1.6		x0.05	
D*A(C)(mg/person*kg a.s.)	0.06			
I*M(mg/person*a.s.)	0.0006			
I*A(mg/person*kg a.s.)	0.001			
ESTIMATION OF OPERATOR EXPOSURE				
	No protection		PPE	
Mixing				
DM(H)	0.648		0.00648	
IM	0.000162		0.000162	
Application				
DA(H)	0.1026		0.1026	
DA(B)	0.432		0.0216	
DA(C)	0.0162		0.0162	
IA	0.00027		0.00027	
RISK ASSESSMENT				
DM(H)/Dtol	0.00925		0.000926	
DA(H)/Dtol	0.01466		0.01466	

DA(B)/Dtol	0.00617	0.00309
DA(C)/Dtol	0.00231	0.00231
IM/Itol	2.31 E-05	2.31 E-05
IA/Itol	3.86 E-05	3.86 E-0.5
Degree of exposure (E)	**0.1718**	**0.0210**