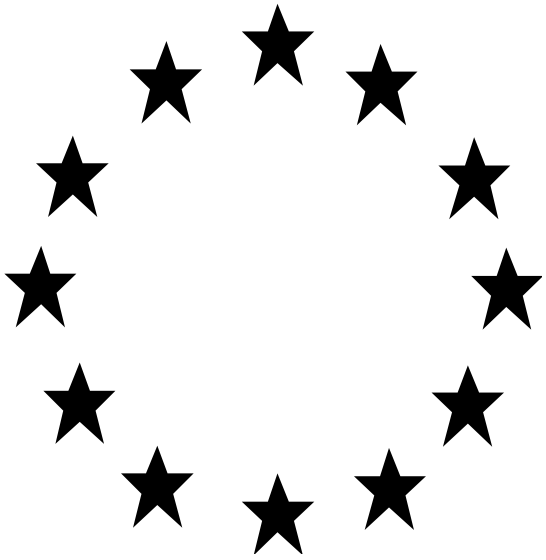

Draft Assessment Report (DAR)

- public version -

Initial risk assessment provided by the rapporteur Member State Sweden for the existing active substance

TOLCLOFOS-METHYL

of the second stage of the review programme referred to in Article 8(2) of Council Directive 91/414/EEC

Volume 3, Annex B, B.6, part 2

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B.6.4 Genotoxicity (Annex IIA 5.4)

B.6.4.1 *In vitro* studies

B.6.4.1.1 Bacterial assay for gene mutation study (1st study)

Reference	: Moriya, M. et al., 1981	**Exposure**	: 24 h (rec-assay), 72 h (reverse mutation test)
Title of study	: S-3349: Microbial mutagenicity study	**Dose**	: Rec-assay: 20, 50, 100, 200, 500, 1000, 2000, 5000 µg/disk; reverse mutation test: 10, 50, 100, 500, 1000, 5000 µg/plate
Test substance	: Tolclofos-methyl, batch No.: not specified, purity: 98.7%, specification No. 01	**Solvent**	: DMSO
Exposure way	: *In vitro*	**GLP statement**	: No
Species	: *Bacillus subtilis, Salmonella typhimurium*	**Guideline**	: In-house method, in accordance with 92/69/EEC, B.14
Group size	:	**Acceptability**	: Yes
		Conclusion	: Not mutagenic

Materials and methods

- Rec. assay: *Bacillus subtilis* H17 (rec^+) and M45 (rec^-) cultures were treated with tolclofos-methyl in dimethyl sulfoxide (0.02 ml per disk) at dose levels indicated above. Positive control (mitomycin C, 0.1 µg/disk), negative control (DMSO) and reference control (kanamycin, 10 µg/disk) were also tested. Each bacterial cells were streaked on an agar plate and a filter paper disk soaked with test chemical solution was placed at the edge of bacterial streaks. The plate was incubated overnight at 37°C. After overnight incubation at 37°C, the length of the inhibitory zones was measured. Results were judged positive when the length of the inhibitory zone of M45 was 3 mm or more at doses that caused 0-1 mm of the inhibitory zone of H17.
- Reverse mutation test: *Salmonella typhimurium* (strains TA1535, TA1537, TA1538, TA98 and TA100) and *Escherichia coli* (WP2 uvrA) cultures were treated with tolclofos-methyl dissolved in dimethyl sulfoxide (0.1 ml/plate) at dose levels indicated above with and without S9 mix, a metabolic activator. Negative control (DMSO) and positive controls were also tested with and without metabolic activation:

2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide 0.01, 0.04, 0.1 µg/plate
N-ethyl-N'-nitro-N-nitrosoguanidine 10 µg/plate
9-aminoacridine 80 µg/plate
2-nitrofluorene 2 µg/plate
2-aminoanthracene 0.5, 2, 40 µg/plate

Direct plate incorporation method; the test chemical solution, each bacterial cell suspension and either phosphate buffer or S9 mix were mixed. After addition of top agar, the mixture was poured onto minimal glucose agar plate. Plates were incubated for 2 days at 37°C. Two plates were used for each treatment. After incubation at 37°C for 2 days, the number of revertant colonies per plate was counted. Results were judged positive when increases in the number of revertants with a dose-response relationship and reproducibility were observed.

Findings

Rec-assay:

The test chemical did not cause any inhibitory zone in either strain even at the highest dose of 5000 µg/disk. The reference control induced similar lengths of inhibitory zones in H17 and M45, while the positive control caused a marked inhibitory zone in M45, compared with that of H17.

Reverse mutation test:

The test chemical induced no increases in the number of revertant colonies of any strain at any dose, compared with those of the corresponding negative control, whether the S-9 mix was added or not. The positive controls were mutagenic for all strains tested.

Conclusions and comments Tolclofos-methyl was negative both in the Rec-assay and in the reverse mutation test with or without metabolic activation. Tolclofos-methyl is non-mutagenic for bacteria under the conditions used in this experiment.

B.6.4.1.2 Bacterial assay for gene mutation study (2nd study)

Reference	: Suzuki, H., Miyamoto, J., 1995	**Exposure**	**:** Rec assay: 24 h, Ames test: 48 h, Host mediated assay: 1h, 2h and 48 h (see materials and methods)
Title of study	: Studies on mutagenicity of S-3349 with bacterial systems	**Dose**	: Rec-assay: 1, 10, 100, 1000 µg/disk; reverse mutation test: 10, 100, 500, 1000, 2000 µg/plate
Test substance	: Tolclofos-methyl, batch No.: not specified, purity: 97.0%, specification No. 01	**Solvent**	: DMSO
Exposure way	: *In vitro*	**GLP statement**	: No
Species	: *Bacillus subtilis, Salmonella typhimurium*	**Guideline**	: In-house method, in accordance with 92/69/EEC, B.14
Group size	:	**Acceptability**	: Yes
		Conclusion	: Not mutagenic

Materials and methods

- Rec. assay: *Bacillus subtilis* H17 (rec^+) and M45 (rec^-) cultures were treated with tolclofos-methyl dissolved in dimethyl sulfoxide (DMSO) at dose levels indicated above. Positive control (*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) 1, 10, 100 µg/disk) and negative control (DMSO 10000 µg/disk) were also tested. Each bacterial cells were inoculated on broth plate and the test chemical solution was applied on a paper disk. After incubation at 37°C for 24 hours, the diameter of growth inhibition zone was measured. Three plates were used for each treatment.
- Reverse mutation test: *Salmonella typhimurium* (strains TA100, TA1535, TA1538, TA98 and TA1537) cultures were treated with tolclofos-methyl in DMSO at dose levels indicated above with and without S9 mixture, a metabolic activator. Positive controls (MNNG 10,100 µg/plate and 2-acetylaminofluorene, 10, 100

µg/plate) and negative control (DMSO) were also tested with and without metabolic activator. The test chemical solution, each bacterial cell suspension and either phosphate buffer or S9 mixture were mixed. The mixture was poured onto minimal plate. After incubation at 37°C for 48 hours, the number of revertant colonies per plate was counted. Three plates were used for each treatment.

- Host-mediated assay: One male ICR mouse per dosing group (body weight: 30 g) was used as the host and was administered orally with tolclofos-methyl (Batch No.: Not specified, Purity: 97.0%, Specification No. 01 - Document J), at dose levels of 870 and 1750 mg/kg. After one hour, about 5 x 10^8 cells of *Salmonella typhimurium* G46 strain used as the indicator organism were injected to the mouse intraperitoneally. Two hours later of post-injection, 1 ml of saline was injected and the indicator cells were harvested. The indicator cells were plated and incubated. Positive control (dimethylnitrosamine 50 and 100 mg/kg) and vehicle (corn oil 3000 mg/kg) were also tested. The number of the mutant and the survival were measured by the plating method.

Findings

Rec-assay:

The test chemical did not cause any inhibitory effect on the growth of either M45 or H17 strains. Tolclofos-methyl was judged to be negative in Rec-assay.

Reverse mutation test:

The number of revertant colonies appearing on the plates treated with tolclofos-methyl was similar to that of the negative controls, in the presence or absence of mammalian metabolizing enzymes.

Host-mediated assay:

The mutation rate of the bacteria recovered from tolclofos-methyl treated mouse abdominal cavity was of the same order as that of the control. No mutagenic effect of the test chemical was observed.

Conclusions Tolclofos-methyl did not show any mutagenic potential either in the Rec-assay, the reverse mutation test, or the host-mediated assay under the test conditions.

B.6.4.1.3 Test for chromosomal aberration test of Rizolex in Chinese hamster ovary cells (CHO-K1)

Reference	: Kogiso, S., 1990	**Exposure**	: 18 and 24 h (without metabolic activation), 2 h (with metabolic activation)
Title of study	: In vitro chromosomal aberration test of Rizolex in Chinese hamster ovary cells (CHO-K1)	**Dose**	: 0, 10, 20 40 µg (without metabolic activation); 37.5, 75, 150 µg/ml (with metabolic activation)
Test substance	: Tolclofos-methyl, batch No.: 90437-M, purity: 96.6%, specification No. 01	**Vehicle**	: DMSO
Exposure way	: *In vitro*	**GLP statement**	: Yes
Species	: Chinese hamster ovary cells (CHO-K1)	**Guideline**	: In-house method, in accordance with 92/69/EEC, B.10
Group size	:	**Acceptability**	: Yes
		Conclusion	: Not mutagenic

Materials and methods

A preliminary cytotoxicity test was performed to determine dose levels to be used and optimal harvest times. The dose levels ranged from 0.6 to 300 µg/ml diluted with a factor of 2 with and without metabolic activation. In the chromosomal aberration test, Chinese hamster ovary cells (CHO-K1) were treated with tolclofos-methyl in dimethyl sulphoxide at dose levels of 10, 20 and 40 µg/ml for 18 and 24 hours in the absence of metabolic activation. In the presence of metabolic activation, dose levels were 37.5, 75 and 150 µg/ml for 2 hours, with further culture in a fresh medium for 16 and 22 hours after removal of the test chemical. The cells were harvested, fixed, stained and analyzed in a blind manner.

Positive controls (MMC: mitomycin C, and CP: cyclophosphamide), untreated control and solvent control were also tested in both tests. Mitotic indices were determined, as well as the number of structural aberrations and the frequencies of cells with structural aberrations, by observation of at least 100 cells from each duplicate culture at each experimental point.

Findings

Cytotoxicity test:

In the absence of metabolic activation, a marked cell cycle delay was observed at 37.5 µg/ml, as well as a moderate decrease in mitotic index (3.1% against 7.5% in the solvent control). In the presence of metabolic activation, a marked cell cycle delay was observed at 150 µg/ml, but no decrease in mitotic index was observed.

Chromosomal aberration test:

The test chemical did not induce any significant increases in the number of total chromosome aberrations nor in the frequency of cells with chromosomal aberrations in the presence or absence of metabolic activation, when compared with those of the corresponding solvent controls. Positive control chemicals induced marked increases both in the number of total chromosome aberrations and in the frequency of cells with chromosomal aberrations.

Conclusions Tolclofos-methyl did not induce chromosomal aberrations in CHO-K1 cells under the conditions used.

B.6.4.1.4 Test for gene mutation in mammalian cells: *In vitro* gene mutation test in Chinese hamster cells (V79) and unscheduled DNA synthesis (UDS) test in HeLa cells

Reference	: Monaco, M., Nunziata, A., 1981	**Exposure**	: 3 h (gene mutation test), 2 h (UDS test)
Title of study	: Report of mutagenicity experiment performed on the test substance S-3349 of the Sumitomo Chemical Company, Limited of Osaka (Japan)	**Dose**	: $5x10^{-6}$, $5x10^{-7}$, $5x10^{-8}$, $5x10^{-9}$ M (gene mutation test); 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} (USD test)
Test substance	: Tolclofos-methyl, batch No.: not specified, purity: not specified, specification No. 01	**Vehicle**	: Acetone
Exposure way	: *In vitro*	**GLP statement**	: No
Species	: Chinese hamster lung cells (V79), HeLa cells	**Guideline**	: In-house method, in accordance with 88/302/EEC, part B
Group size	:	**Acceptability**	: Yes
		Conclusion	: Not mutagenic

Materials and methods

- Gene mutation test: Chinese hamster lung cells (V79) were treated with tolclofos-methyl in acetone at dose levels indicated above with and without metabolic activation (S9 mix). Negative (untreated and acetone) and positive controls (methyl methanesulfonate 10^{-5} M and dimethylnitrosamine 10^{-5} M) were also tested. After incubation for 3 hours at 37°C, the cells were washed and cultured at 37°C until confluence. At each expression time (24, 72, 120 and 168 hours), a portion of cells was inoculated to dishes with or without 6-thioguanine and further cultivated to determine mutation frequency and plating efficiency, respectively. The number of mutant colonies and the plating efficiency were determined and mutation frequency was calculated at each expression time.
- UDS test: Human cervical carcinoma cells (HeLa cells) were treated with tolclofos-methyl in acetone at dose levels of indicated above with and without metabolic activation (S9 mix). Positive controls (methyl-methanesulfonate 10^{-5} M and urethane 10^{-5} M) were also tested. After incubation for 2 hours at 37°C, cells were washed and treated with 5µCi/ml of tritiated thymidine for 4 hours at 37°C. These cells were fixed and the slides with cells on them were subjected to autoradiographic process and stained in Giemsa solution. The number of grains, caused by the incorporation of tritiated thymidine to DNA, in the nucleus and in an equivalent extracellular region was counted under a microscope. Fifty cells were counted for each slide.

Findings

Gene mutation test:

The test substance did not induce significant increase in the mutation frequency of the treated cells, when compared with the negative controls.

UDS test:

The test substance did not induce any statistically significant increase in the unscheduled DNA synthesis in the treated cells, when compared with the vehicle controls. The positive controls showed distinctly significant values.

Conclusions No increase in the mutation frequencies or in the number of net grain counts was observed. Tolclofos-methyl did not induce gene mutation in mammalian cells or *in vitro* unscheduled DNA synthesis under the test conditions used.

B.6.4.2 *In vivo* studies and further *in vitro* studies using different metabolizing systems

B.6.4.2.1 *In vivo* chromosomal aberration test on bone marrow cells of mice

Reference	: Suzuki, H., 1981	**Exposure**	: Single injection
Title of study	: *In vivo* chromosomal aberration test of S-3349 on bone marrow cells of mice	**Dose**	: 500, 1000, 2000, 4000 mg/kg bw
Test substance	: Tolclofos-methyl, batch No.: 00304, purity: 99.8%, specification No. 01	**Vehicle**	: Corn oil (5 ml/kg bw)
Exposure way	: Intraperitoneal	**GLP statement**	: No
Species	: ICR mice	**Guideline**	: In-house method, in accordance with 92/69/EEC, B.11
Group size	: 6/dose (males)	**Acceptability**	: Yes (with comments)
		Conclusion	: Not clastogenic

Materials and methods

Groups of 6 male ICR mice each received a single intraperitoneal injection of tolclofos-methyl in suspension in corn oil at dose levels indicated above. Mice were injected with 4 mg/kg of colchicine 2 hours before sacrifice. Mice were sacrificed 6 and 24 hours after single doses of 1000, 2000 and 4000 mg/kg bw, and 48 hours after treatment of 500 and 1000 mg/kg bw. In the 2000 and 4000 mg/kg groups, 5 and 3 out of 6 mice were dead after 48 hours treatment and slide preparations were not made. Bone marrow cells were then harvested, fixed, stained (Giemsa) and analyzed in a blind manner. Fifty well-spread metaphases from each animal were observed under microscope. Positive control (cyclophosphamide 60 mg/kg) was also carried out (6, 24 and 48 hr treatments).

Findings

In the tolclofos-methyl treated groups, there was no significant increase in the frequency of chromosomal aberrations in exception of the 48 hr treated group of 1000 mg/kg, in which the frequency of aberrant cells was 1.7% (5/300), while 0.0% (0/300)of the cells in the corresponding vehicle control group ($p < 0.05$) showed chromosomal aberrations. The positive control (cyclophosphamide 60 mg/kg) produced significant amount of chromosomal aberrations (Table B.6.4.2.1-1).

Table B.6.4.2.1-1: *In vivo* chromosomal aberrations in bone marrow cells: Frequency of aberrations

Chemicals	Dose (mg/kg)	Time after injection (hr)	No. of animals	No. of analyzed cells	Cell with aberration (%)	No. of aberrations[a]				
						G	B	E	m.a.	P
Corn oil	-[b]	6	6	300	0.3	1	0	0	0	0
Tolclofos-methyl	1000	6	6	300	1.3	2	2	0	0	0
	2000	6	6	300	1.0	2	1	0	0	0
	4000	6	6	300	1.0	3	0	0	0	0
CP	60	6	4	200	7.5**	13	5	0	0	0
Corn oil	-	24	6	300	1.7	4	1	0	0	0
Tolclofos-methyl	1000	24	6	300	1.0	3	0	0	0	0
	2000	24	6	300	1.0	1	2	0	0	0
	4000	24	6	300	2.3	6	1	0	0	0
CP	60	24	4	200	28.0**	26	15	1	9	22
Corn oil	-	48	6	300	0.0	0	0	0	0	0
Tolclofos-methyl	500	48	6	300	1.0	3	0	0	0	0
	1000	48	6	300	1.7*	3	2	0	0	0
CP	60	48	4	200	7.5**	2	0	0	4	9

a: Number of aberrations catergorized as follows;
b: 10 ml/kg bw
G: Gaps, B: Breaks, E: Exchanges, P: Pulverisation of chromosomes,
m.a.: Cells with multiple aberrations (>9)
CP: Cyclophosphamide
**: $p < 0.05$*
***: $p < 0.01$*

Conclusion and comments

Tolclofos-methyl is not considered to be clastogenic with respect to chromosomal aberrations in bone marrow cells of mice. There was however a significant, albeit small, increase in aberrations in the 1000 mg/kg bw group 48 h post treatment. The biological significance is, however obscure, since the historical control of this laboratory is in the range of 0.3 – 2%. It would have been interesting to see data from metaphase preparations from the surviving animals in the 2000 and 4000 mg/kg bw groups. Furthermore, the choice of dose levels can be questioned. It is known from earlier studies (Segawa, T., 1978, see 6.2.1.1 Acute studies) that dead animals are expected at doses of 2000 and 4000 mg/kg bw/day.
The results are, however, considered clear and no repeat experiments are required.

B.6.4.2.2 *In vitro* unscheduled DNA synthesis (UDS) in rat hepatocytes

Reference	: Hara, M., 1990	**Exposure**	: 18 h
Title of study	: *In vitro* unscheduled DNA synthesis (UDS) in rat hepatocytes	**Dose**	: 0.3, 1, 3, 10, 20, 40 µg/ml
Test substance	: Tolclofos-methyl, batch No.: 90437-M, purity: 96.6%, specification No. 01	**Vehicle**	: DMSO
Exposure way	*: In vitro*	**GLP statement**	: Yes
Cell type	: Rat hepatocytes	**Guideline**	: In-house method, in accordance with 88/302/EEC , Part B
Group size	: 5/sex/dose	**Acceptability**	: Yes
		Conclusion	: Not DNA damaging

Material and methods

Hepatocytes were isolated from male 7 week-old Sprague-Dawley rats. A preliminary cytotoxicity test was performed to determine dose levels to be used in the UDS assay. The dose levels ranged from 4.7 to 300 µg/ml. In the UDS test, the hepatocyte primary culture was treated with tolclofos-methyl at the concentrations indicated above at 37°C in presence of ^{3}H-thymidine. ^{3}H-thymidine incorporated into hepatocyte DNA was detected as grains by using an autoradiographic technique. Positive control (2-acetylaminofluorene) and solvent control were also tested. The test was conducted in duplicate and repeated twice using hepatocytes from different rats.

Findings

Cytotoxicity test:

The compound showed a dose-related cytotoxicity on hepatocytes and precipitated at 150 and 300 µg/ml. From these results, a concentration of 40 µg/ml, at which about 50% of viability was expected, was selected as the highest dose for the UDS assay.

Unscheduled DNA assay:

In test 1, tolclofos-methyl showed a statistically significant increase in net grains (NG) at 3 µg/ml, but there was no dose-related increase or reproducibility in NG. The percentages of cells in repair in the treated groups were comparable to the vehicle control values. In test 2, neither NG nor cells in repair in the treated groups differed from those of the vehicle control group (Tables B.6.4.2.2-1A and 1B). The positive control chemical induced marked increases in both net grain counts and the percentage of cells in repair.

Table B.6.4.2.2-1A: UDS test in mammalian cells: Assessment of grains of DNA incorporation (Test 1)

Chemicals	Dose (µg/ml)	Viability (%)	NG[a]	Nuclear grain	Cytoplasmic grain	Cells in repair[b]
Test 1						
DMSO[c]	-	100	- 12.8	25.8	38.6	0/50
			- 13.8	24.5	38.3	0/50
		Mean	*- 13.3*	*25.2*	*38.5*	*0/100*
Tolclofos-methyl	0.3	101.1	- 10.8	19.8	30.7	0/50
			- 13.4	24.0	37.5	0/50
		Mean	*- 12.1*	*21.9*	*34.1*	*0/100*
	1	107.6	- 13.9	21.4	35.2	0/50
			- 11.8	18.7	30.5	0/50
		Mean	*- 12.9*	*20.1*	*32.9*	*0/100*
	3	105.1	- 9.5	33.7	43.2	0/50
			- 8.2	30.6	38.9	2/50
		Mean	*- 8.9**	*32.2**	*41.1*	*2/100*
	10	89.3	- 14.2	26.2	40.3	0/50
			- 13.1	24.2	37.3	1/50
		Mean	*- 13.7*	*25.2*	*38.8*	*1/100*
	20	70.2	Toxic	Toxic	Toxic	-
			- 9.0[NT]	24.3[NT]	33.2	1/50
		Mean	-	-	-	*1/50*
	40	4.3	Toxic	Toxic	Toxic	-
			Toxic	Toxic	Toxic	-
		Mean	-	-	-	-
2-AAF	0.05	103.9	27.2	66.5	35.3	49/50
			27.9	58.9	26.3	50/50
		Mean	*27.6***	*62.7**	*30.8*	*99/100[#]*

a: The mean of net grain counts, 50 cells were counted from each slide

b: Number of cells with 5 NG or more
c: Dimethyl sulfoxide. Final concentration in medium was 1%
2-AAF: 2-Acetylaminofluorene
Toxic: Sufficient number of analyzable cells were not obtained because of cytotoxicity
**: p < 0.05[Student's t test]*
***: p < 0.01 [Student's t test]*
NT: Student's t test was not applied between the vehicle control and the tolclofos-methyl 20 µg/ml treated group
#: 20% or more cells in repair

Table B.6.4.2.2-1B: UDS test in mammalian cells: Assessment of grains of DNA incorporation (Test 2)

Chemicals	Dose (µg/ml)	Viability (%)	NG[a]	Nuclear grain	Cytoplasmic grain	Cells in repair[b]
Test 2						
DMSO[c]	-	100	- 7.1	36.4	43.4	0/50
			- 11.5	20.3	31.7	0/50
		Mean	*- 9.3*	*28.4*	*37.6*	*0/100*
Tolclofos-methyl	0.3	98.5	- 11.4	21.1	32.5	0/50
			- 13.8	21.3	35.1	0/50
		Mean	*- 12.6*	*21.2*	*33.8*	*0/100*
	1	98.7	- 10.4	25.6	36.0	2/50
			- 11.5	24.4	36.0	0/50
		Mean	*- 11.0*	*25.0*	*36.0*	*2/100*
	3	98.0	- 9.3	30.3	39.6	0/50
			- 11.9	24.5	36.4	0/50
		Mean	*- 10.6*	*27.4*	*38.0*	*0/100*
	10	70.0	- 5.6	37.9	43.5	2/50
			- 9.5	26.4	35.9	1/50
		Mean	*- 7.6*	*32.2*	*39.7*	*3/100*
	20	49.2	- 8.2	23.4	31.6	0/50
			- 4.9	19.0	23.9	1/50
		Mean	*- 6.6*	*21.2*	*27.8*	*1/100*
	40	21.8	Toxic	Toxic	Toxic	-
			Toxic	Toxic	Toxic	-
		Mean	-	-	-	-
2-AAF	0.05	97.7	20.8	62.9	34.1	49/50
			19.3	57.8	34.9	46/50
		Mean	*20.1***	*60.4*	*34.5*	*95/100#*

a: The mean of net grain counts, 50 cells were counted from each slide
b: Number of cells with 5 NG or more
c: Dimethyl sulfoxide. Final concentration in medium was 1%
2-AAF: 2-Acetylaminofluorene
Toxic: Sufficient number of analysable cells were not obtained because of cytotoxicity
**: p < 0.05[Student's t test]*
***: p < 0.01 [Student's t test]*
NT: Student's t test was not applied between the vehicle control and the tolclofos-methyl 20 µg/ml treated group
#: 20% or more cells in repair

Conclusions Tolclofos-methyl did not induce UDS and has no DNA-damaging activity on rat hepatocyte primary cultures.

B.6.4.2.3 In vivo studies in germ cells: Rat dominant lethal assay

Reference	: Brusick, D.J., 1981	**Exposure**	**:** 5 days
Title of study	: Mutagenicity evaluation of S-3349 T.G. lot No. 4 in the rat dominant lethal assay	**Dose**	: 62.5, 208.3, 625 mg/kg bw/day
Test substance	: Tolclofos-methyl, batch No.: 4, purity: not specified, specification No. 01	**Vehicle**	: Corn oil (1.9 ml)
Administration way	: Oral *via* gavage	**GLP statement**	: No, but performed according to GLP of US Food and Drug Administration
Species	: CRL:COBS CD (SD)BR Sprague-Dawley rats	**Guideline**	: In-house method, in accordance with 88/302/EEC , Part B
Group size	: 10/sex/dose	**Acceptability**	: Yes
		Conclusion	: Did not produce dominant lethal effects

Materials and methods

Tolclofos-methyl was administered at dose levels selected from acute oral LD_{50} (5000 mg/kg). The high dose was set at 1/8 LD_{50} and the mid and low doses were set at 1/3 and 1/10 the high dose respectively.). The positive control group (TEM: triethylenemelamine) received a single administration of 0.3 mg/kg (volume of administration: 0.1 ml/animal) by the intraperitoneal route on day 5 of the dosing schedule. After the male rats were exposed to the test material they were mated sequentially to virgin females (two females per week for 7 weeks). The females were killed at mid-pregnancy (14 days after the mid-week of mating). At necropsy the uteri were examined and the number of corpora lutea and living and dead implantations were counted for each pregnant female. The fertility index, the total number of implantations, the total number of corpora lutea, preimplantation losses, dead implantations, the proportion of females with one (two) or more dead implantations and dead implants/total implants were calculated and evaluated. Statistical analysis was used for the evaluation of the results (chi-square test and Armitage's trend test (fertility index), Student's t-test (total number of implantations, total number of corpora lutea, dead implants/total implants), Student's t-test and regression analysis (preimplantation losses, dead implantations), chi-square test, Armitage's trend test and probit regression analysis (proportion of females with one (two) or more dead implantations)).

Findings

Fertility:

There was no reduction in fertility over the dose range of tolclofos-methyl employed. The range of implants per pregnant female was very consistent over all groups except for the positive control group: females mated to positive control group males showed significantly lower number of implant over the first four weeks. There were several instances in which the number of corpora lutea counted per pregnant female was lower than the corresponding negative control. There were no clear trends in the reductions by dose or by mating week. The values were not significantly lower than the historical controls. Most preimplantation losses were equal to or lower than the controls indicating no effect from the test material. Only minor increases in preimplantation loss were observed and these did not appear dose-related and were associated with unusually low concurrent negative control value (Table B.6.4.2.3-1).

Table B.6.4.2.3-1: Rat dominant lethal test: Fertility data

Week	Historical Negative Control	Negative Control	Positive Control	Tolclofos-methyl		
				62.5 mg/kg	208.3 mg/kg	625.0 mg/kg
Fertility index						
1	0.44	0.80	0.80	0.75	0.80	0.85
2	0.52	0.85	0.95	0.85	0.95	0.85
3	0.57	0.85	0.75	0.90	0.85	0.90
4	0.64	0.75	0.80	0.80	0.95	0.80
5	0.63	0.90	0.80	0.80	0.95	0.95
6	0.64	0.85	0.85	0.80	0.85	0.90
7	0.67	0.80	1.00	0.80	0.95	1.00
Average number of implantations per pregnant female						
1	12.03	13.25	10.69*	14.0	13.75	14.41
2	11.68	12.65	8.84**	13.18	13.79	12.76
3	11.85	13.00	5.73**	13.29	12.94	13.22
4	12.92	13.33	5.94**	13.06	13.47	13.06
5	12.66	13.33	11.69	13.44	14.00	13.37
6	11.92	13.47	12.47	14.00	13.65	14.17
7	12.81	14.81	13.30	13.75	14.79	13.55
Average corpora lutea per pregnant female						
1	15.81	19.31	18.44	19.73	16.62	16.94
2	15.28	18.94	19.21	15.94*	17.68	15.29**
3	14.96	15.17	13.60	18.83**	15.06	18.00*
4	15.76	18.47	16.44	15.44	16.84	14.50*
5	16.34	17.44	17.19	14.06**	18.16	15.00**
6	15.13	14.94	13.47	19.88**	15.18	18.56**
7	15.58	16.75	16.90	19.12	16.21	21.35**
Average preimplantation losses per pregnant female						
1	3.78	6.06	7.75	5.67	2.88*	2.53**
2	3.60	6.29	10.37**	2.76*	3.90*	2.53**
3	3.11	2.18	7.87**	5.56**	2.12	4.78*
4	2.84	5.13	10.50**	2.38	3.37	1.44*
5	3.68	4.11	5.50	0.62**	4.16	1.63*
6	3.20	1.47	1.00	5.88**	1.53	4.39*
7	2.77	1.94	3.60	5.38**	1.42	7.80**

**: Significantly different from control ($p < 0.05$); **: Significantly different from control ($p < 0.01$)*

Embryo lethality:

No evidence for embryo lethality associated with the test compound was seen. The parameter of dead implants shows dominant lethal activity and the effects on this parameter were observed only in the females mated to the positive control group. The following indices showed no effect in the treated groups whereas the positive control substance was active in inducing dominant lethality: proportion of females with one or more and two or more dead implants, number of dead implants/total number of implants, number of living implants/pregnant female.

Conclusions There were no dominant lethal effects induced by tolclofos-methyl in rats under the conditions used in the study. The deviations in preimplamantation losses did not show a dose-response.

B.6.4.3 Summary and conclusions on genotoxicity testing

Study	Dose levels	Result
Moriya et al., 1981 Mutagenicity assay in *Salmonella typhimurium & Escherichia coli*	0, 10, 50, 100, 500, 1000, 5000 µg/plate, +/- S9 mix	Negative
Suzuki et al., 1978 Mutagenicity assay in *Salmonella typhimurium*	0, 10, 100, 500, 1000, 2000 µg/plate, +/- S9 mix	Negative
Moriya et al., 1981 Rec-assay in *Bacillus subtilis* H17 & M45	0, 20, 50, 100, 200, 500, 1000, 2000, 5000 µg/disk	Negative
Suzuki et al., 1978 Rec-assay in *Bacillus subtilis* H17 & M45	0, 1, 10, 100, 1000 µg/disk	Negative
Suzuki et al., 1978 Host-mediated assay, male ICR mouse injected with *Salmonella typhimurium* G46	0, 870, 1750 mg/kg	Negative
Monaco et al., 1981 Gene mutation assay in Chinese hamster lung cells (V79	$5x10^{-6}$, $5x10^{-7}$, $5x10^{-8}$, $5x10^{-9}$ M, +/- S9 mix	Negative
Kogiso et al., 1990 *In vitro* chromosomal aberration test in Chinese hamster ovary cells (CHO-K1)	0, 37.5, 75, 150 µg/ml (+S9, 2+16 and 2+22 h) 0, 10, 20, 40 µg/ml (-S9, 18 and 24 h)	Negative
Monaco et al. 1981 *In vitro* unscheduled DNA synthesis test in human cervical carcinoma cells (HeLa)	$1x10^{-6}$, $1x10^{-7}$, $1x10^{-8}$, 10^{-9} M, +/- S9 mix	Negative
Hara et al., 1990 *In vitro* unscheduled DNA synthesis test in rat primary hepatocytes	0, 0.3, 1, 3, 10, 20, 40 µg/ml (18 h)	Negative
Suzuki et al., 1981 *In vivo* chromosomal aberration test of S-3349 on bone marrow cells of mice	0, 1000, 2000, 4000 mg/kg (6 and 24 h) 0, 500, 1000 mg/kg (48 h)	Negative
Brusick et al., 1981 *In vivo* study in germ cells: dominant lethal assay, male CD(SD)BR Sprague-Dawley rats	0, 62.5, 208.3, 625 mg/kg daily for 5 days	Negative

The mutagenic potential of tolclofos-methyl was studied *in vitro* in bacteria and mammalian cells and *in vivo* test systems in somatic cells and in germ cells. The test systems assayed did not show evidence of tolclofos-methyl genotoxicity. Overall, the studies indicate that tolclofos-methyl does not possess any concern for genotoxicity.

B.6.5 Long-term toxicity and carcinogenicity (Annex IIA 5.5)

B.6.5.1 Chronic toxicity and carcinogenicity study in rats

Reference	: Pence, D. H. et al., 1982, 1983; Seki, T. et al., 1985	**Exposure**	: 122 weeks (males), 129 weeks (females)
Title of study and amendments	: Chronic toxicity in rats – S-3349: Final report addendum (Pence et al.); Comments on toxic effects and minimum effect level of S-3349 in chronic oral toxicity studies in rats (Seki et al.)	**Dose**	: 0, 100, 300, 1000 ppm equivalent to 0, 4.2, 12, 42 mg/kg bw/day (males) 0, 4.8, 15, 49 mg/kg bw/day (females)
Test substance	: Tolclofos-methyl, batch No.: 523, purity: 94.9%, specification No. 02; batch No.: 4, purity: 98.7%, specification No. 01	**Vehicle**	: Diet
Administration way	: Oral *via* the diet	**GLP statement**	: No, but study performed according to GLP for US Food and Drug Administration
Species	: Fischer 344 CD®F rats	**Guideline**	: In-house method, in accordance with 88/302/EEC , Part B
Group size	: 65/sex/dose	**Acceptability**	: Yes
		NOAEL/ NOEL	: ≥1000 ppm (42 mg/kg bw/day)

Materials and methods

Samples were taken from 10 animals/sex/group at weeks –1, 4, 13, 26, 52, 78 and 104, 10 males/group at week 122 and 10 females/group at week 129 for haematology, clinical chemistry and urinalysis evaluations. Ophthalmology evaluations were performed at weeks 26, 52, 78 and 104, on males at week 122 and on females at week 129. Body weight was measured once a week for the first 26 weeks, biweekly for the succeeding 26 weeks and every four weeks for the remainder of the study. All animals sacrificed by design, dead or killed in extremis during the study were subjected to gross pathology and histopathology.

Findings

General observations:

Deaths were noted during the study but there was no compound effect upon mortality. No distinct clinical signs of compound effect were observed for any of the treatment levels during the study. During the terminal observation digital palpation revealed the presence of palpable nodules, masses, and/or wart-like lesions in few animals of the control and treated groups. No signs of compound effect were discernible with regard to body weight and food consumption data. Water consumption was consistently higher than control in the high-dose males at all of the recording intervals and was occasionally higher than control in the low- and/or mid- dose males at some of the intervals.

Haematology, clinical chemistry, urinalysis and cholinesterase activity:

Results of the haematology examinations and urinalysis did not reveal any distinct compound-related changes. At week 52, brain cholinesterase (ChE) values were decreased in several of the compound-treated groups. (Table

B.6.5.1-1). At termination of the study (2 years), brain, plasma and erythrocytes values were generally comparable to control values.

Alkaline phosphatase values were reduced in many of the male and female compound-treated groups (Table B.6.5.1-2).

Table B.6.5.1-1: Chronic toxicity study in rats: Cholinesterase activity (Δ pH/min)

Dose level	0 ppm	100 ppm	300 ppm	1000 ppm
Males				
Erythrocytes				
Week -1	1.05	1.10	0.90	1.00
Week 52	0.86	0.87	0.84	0.82
Week 122	0.60	0.64	0.68	0.61
Plasma				
Week -1	0.81	0.78	0.78	0.79
Week 52	0.82	0.78	0.64	0.71
Week 122	1.20	1.19	1.40	1.32
Brain				
Week 52	1.27	0.48 *	0.91	0.85
Week 122	1.26	1.31	0.60	1.32
Females				
Erythrocytes				
Week -1	0.87	0.84	0.80	0.81
Week 52	0.77	0.77	0.77	0.77
Week 129	0.75	0.82	0.77	0.84
Plasma				
Week -1	1.28	1.37	1.29	1.30
Week 52	2.36	2.36	2.35	2.29
Week 129	1.90	1.64	1.76	1.96
Brain				
Week 52	1.22	0.58	0.24*	0.38*
Week 129	1.50	0.65	0.77	1.35

**: Significantly different from control ($p < 0.05$)*

Table B.6.5.1-2: Chronic toxicity study in rats: Clinical chemistry

Dose level	0 ppm	100 ppm	300 ppm	1000 ppm
Alkaline phosphatase (IU/l)				
Males				
Week -1	332	306.9	328.7	301.4
Week 52	82.6	63.1*	59.0*	49.8*
Week 122	84	77	49	45
Females				
Week -1	250.6	235.2	248.4	262.9
Week 52	42.6	46.5	34.7	38.3
Week 129	64	60	57	49

**: Significantly different from control ($p < 0.05$)*

Gross pathology, organ weights and histopathology:

Ophthalmic examinations afforded no evidence for treatment related changes of the eyes. None of the differences observed in organ weights were significant and there was no distinct dose-related pattern. No distinct compound-related organ or tissue changes were observed in any of the animals sacrificed by design or in those that died or were sacrificed in extremis during the study. Histopathological examinations at week 52 revealed a slight higher incidence of interstitial cell tumors in the testes of the control males; these differences were not statistically significant as judged by the chi-square test and Fisher's exact test. At terminal sacrifice as well as in the animals that died or were sacrificed in extremis during the study, the incidence of interstitial cell tumors was

comparable between the compound-treated and control males (Table B.6.5.1-3). Neoplasms, spontaneous disease lesions and incidental findings were consistent with lesions routinely observed in rats of this age and strain (Tables 6.5.1-6A and 6B).

Table B.6.5.1-3: Chronic toxicity study in rats: Incidence of histopathologically proven tumors

Dose level	0 ppm	100 ppm	300 ppm	1000 ppm
Dead and moribund sacrifices				
No. examined	31	26	27	35
Interstitial cell tumors	29	24	26	34
Mesothalioma	1	2	0	2
Terminal sacrifices				
No. examined	23	28	27	19
Interstitial cell tumors	23	28	27	18
Mesothelioma	1	1	1	1

Table B.6.5.1-4A: Chronic toxicity study in rats: Neoplasms classification summary (Dead and moribund sacrifices)

	Dead and moribund sacrifices							
Sex	**Male**				**Female**			
Dose level (ppm)	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Neoplasm classification summary								
Number of animals	32	27	28	36	36	27	34	25
Total primary neoplasms	83	69	84	97	82	53	61	41
Animals with one or more	31	26	27	36	32	25	31	22
Percent with one or more	96 %	96 %	96 %	100 %	88 %	92 %	91 %	88 %
Total begnin neoplasms	51	45	54	66	44	28	37	19
Animals with one or more	30	25	27	36	26	18	23	14
Percent with one or more	93 %	92 %	96 %	100 %	72 %	66 %	67 %	56 %
Total malignant neoplasms	32	24	30	31	38	25	24	22
Animals with one or more	24	17	21	21	25	20	20	16
Percent with one or more	75 %	62 %	75 %	58 %	69 %	74 %	58 %	64 %
Total metastatic neoplasms	8	6	0	2	0	4	1	1
Animals with one or more	2	3	0	2	0	2	1	1
Percent with one or more	6 %	11 %	0 %	5 %	0 %	7 %	2 %	4 %
Total locally invasive neoplasms	2	0	2	1	2	6	3	2
Animals with one or more	2	0	1	1	2	4	2	1
Percent with one or more	6 %	0 %	3 %	2 %	5 %	14 %	5 %	4 %
Total other neoplasms	0	0	0	0	0	0	0	0
Animals with one or more	0	0	0	0	0	0	0	0
Percent with one or more	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %

Table B.6.5.1-4B: Chronic toxicity study in rats: Neoplasms classification summary (Terminal sacrifices)

	Terminal sacrifices							
Sex	**Male**				**Female**			
Dose level (ppm)	**0**	**100**	**300**	**1000**	**0**	**100**	**300**	**1000**
Neoplasm classification summary								
Number of animals	23	28	27	19	19	28	21	30
Total primary neoplasms	80	86	101	60	67	92	60	93
Animals with one or more	23	28	27	19	19	25	21	30
Percent with one or more	100 %	100 %	100 %	100 %	100 %	89 %	100 %	100 %
Total begnin neoplasms	54	60	67	44	41	50	38	62
Animals with one or more	23	28	27	19	18	23	19	28
Percent with one or more	100 %	100 %	100 %	100 %	94 %	82 %	90 %	93 %
Total malignant neoplasms	26	26	34	16	26	42	22	31
Animals with one or more	19	17	23	11	18	24	16	22
Percent with one or more	82 %	60 %	85 %	57 %	94 %	85 %	76 %	73 %
Total metastatic neoplasms	8	4	0	4	1	8	1	0
Animals with one or more	2	1	0	2	1	1	1	0
Percent with one or more	8 %	3 %	0 %	10 %	5 %	3 %	4 %	0 %
Total locally invasive neoplasms	0	0	0	0	1	0	1	0
Animals with one or more	0	0	0	0	1	0	1	0
Percent with one or more	0 %	0 %	0 %	0 %	5 %	0 %	4 %	0 %
Total other neoplasms	0	0	0	0	0	0	0	0
Animals with one or more	0	0	0	0	0	0	0	0
Percent with one or more	0 %	0 %	0 %	0 %	0 %	0 %	0 %	0 %

Conclusions

No distinct signs of compound effect were observed with regard to mortality, clinical signs, body weights, food consumption, organ weights and organ/body weight ratios, and gross pathology. No histomorphological alterations were attributable to the test compound at dietary levels up to 1000 ppm.

Tolclofos-methyl was not oncogenic in this study.

NOAEL/NOEL: ≥ 1000 ppm (42 mg/kg bw/day)

B.6.5.2 Chronic toxicity study in rats (Cholinesterase activity study)

Reference	: Pence D.H. et al., 1985a	**Exposure**	: 104 weeks
Title of study	: 104-week cholinesterase activity study in male and female rats – S-3349	**Dose**	: 0, 100, 300, 1000 ppm, equivalent to 0, 4.1, 12, 42 mg/kg bw/day (males); 0, 4.8, 15, 49 mg/kg bw/day (females)
Test substance	: Tolclofos-methyl, batch No.: 10901, purity: 97.9 and 98.3%, specification No. 01	**Vehicle**	: Diet
Administration way	: Oral *via* the diet	**GLP statement**	: No, but study performed according to GLP for US Food and Drug Administration
Species	: Fischer 344 rats	**Guideline**	: In-house method, in accordance with 88/302/EEC, Part B
Group size	: 30/sex/dose	**Acceptability**	: Yes
		NOAEL/ NOEL	: ≥1000 ppm (42 mg/kg bw/day)

Materials and methods

Samples were taken from 10 animals/sex/group at initiation of the study, at weeks 5, 14, 27, 53, 79 and at termination for blood cholinesterase determination; for brain cholinesterase activity determination, samples were taken at week 53 and at termination. Food consumption and body weights were measured weekly during the first 26 weeks, once every two weeks from weeks 26 through 52 and once every four weeks from weeks 53 through 104. Examination and palpation for incidence and location of tissue masses were performed at each weighing interval. All animals were subjected to gross pathology at necropsy.

Findings

General observations:

Death was noted in few animals in all groups. There were no compound related effects on survival. The incidence of clinical signs and palpable tissue masses was comparable among groups. Mean body weights and food consumption values were comparable among groups throughout the study.

Blood and brain cholinesterase activity:

Mean erythrocyte cholinesterase and brain cholinesterase activities were comparable among groups throughout the study. (Table B.6.5.2-1).

Table B.6.5.2-1: 104-week Cholinesterase activity study in rats: Cholinesterase activity (µmol/ml/min)

Dose level	0 ppm	100 ppm	300 ppm	1000 ppm
Males				
Erythrocytes				
Week 0	2.03	2.03	2.00	2.03
Terminal	2.07	1.97	2.17	1.97
Plasma				
Week 0	1.10	1.13	1.07	1.10
Terminal	2.53	2.03	2.17	1.80
Brain				
Week 53	17.60	16.87	16.67	16.37
Terminal	18.10	17.70	17.10	17.43
Females				
Erythrocytes				
Week 0	1.93	2.00	1.93	1.93
Terminal	2.03	2.27	2.03	2.17
Plasma				
Week 0	1.87	1.60 *	1.73	1.73
Terminal	3.73	4.00	4.43	3.70
Brain				
Week 53	17.23	16.50	17.03	16.43
Terminal	17.63	18.20	17.83	17.97

a: Excluding three artefactually elevated values
**: Significantly different from control ($p < 0.05$)*

Gross pathology: Gross pathology findings for all animals were considered to be incidental and unrelated to compound administration.

Conclusions No treatment-related effects were shown on the parameters examined in this study.
NOAEL/NOEL: ≥1000 ppm (equivalent to 42 mg/kg bw/day).

B.6.5.3 Carcinogenicity study in the mouse: Chronic toxicity study in mice

Reference	: Satoh, T. et al., 1983	**Exposure**	: 24 months
Title of study	: Twenty-four month chronic toxicity study of S-3349 in pulverized diet in mice	**Dose**	: 0, 10, 50, 250, 1000 ppm, equivalent to: 1.3, 6.4, 32.2, 134 mg/kg bw/day (males) and 1.3, 6.9, 34.1, 137 mg/kg bw/day (females)
Test substance	: Tolclofos-methyl, batch No.: not specified, purity: 94.3%, specification No. 02	**Vehicle**	: Pulverised basal diet CE-2
Administration way	: Oral *via* the diet	**GLP statement**	: No
Species	: Crj:B6C3F1 mice	**Guideline**	: In-house method, in accordance with 88/302/EEC , Part B
Group size	: 50/sex/dose (main study), 20/sex/dose (satellite group)	**Acceptability**	: Yes
		NOAEL	: 250 ppm (32.2 mg/kg bw/day)
		NOEL	: 50 ppm (6.4 mg/kg bw/day)

Materials and methods

Samples were taken as follows:

- for urinalysis: from 10 animals/sex/main group at month 18, from all survivals of the main groups at month 24; from 10 animals/sex/satellite group at months 6 and 12;
- for haematology: from 10 animals/sex/satellite group at month 12, from all survivals of the main groups at month 24;
- for clinical chemistry: from 10 animals/sex/satellite group at months 6 and 12, from all survivals of the main groups at month 24.

Body weights were measured: once a week for the first 13 weeks and thereafter once per 4 weeks in the main groups; once per 4 weeks in the satellite groups. Organ weights were measured for the animals sacrificed by design (months 6 and 12) and all survivals after 24 months. Gross pathology was performed on all the animals found dead or sacrificed by design or moribund at months 6, 12 and 24. Histopathological evaluations were performed on all animals.

Findings

General observations:

There was no effect of administration of the test compound on general symptoms, time and number of occurrence of external masses and mortality. Suppression of weight gain after 52 weeks was observed in the female 1000 ppm group (Table B.6.5.3-1). A decrease in food consumption was seen in female 1000 ppm group (Table B.6.5.3-2).

Table B.6.5.3-1: 24-month chronic toxicity in mice: Body weight and body weight gain (g)

Dose level	0 ppm	10 ppm	50 ppm	250 ppm	1000 ppm
Males					
Body weight					
	40.3	38.4	38.6	40.5	41.6
Body weight gain					
Week 0-52	19.69	21.58**	21.32**	20.96	20.23
Week 0-104	21.62	19.85	20.02	21.78	22.78
Females					
Body weight					
	41.8	43.9	41.2	39.8	39.6
Body weight gain					
Week 0-28	14.31	14.14	13.15*	13.68	13.11*
Week 0-52	17.13	17.63	15.80*	15.87*	14.59***
Week 0-104	26.10	28.03	25.69	24.10	23.91

**: p < 0.05 in comparison with controls*
***: p < 0.01 in comparison with controls*
****: p < 0.001 in comparison with controls*

Table B.6.5.3-2: 24-month chronic toxicity in mice: Food consumption

Dose level	0 ppm	10 ppm	50 ppm	250 ppm	1000 ppm
Males					
Week 0-104					
Mean food/day (g)	4.88	4.80	4.78*	4.82	4.91
Total food intake (g)	3561.1	3505.6	3490.4	3519.2	3584.0
Females					
Week 0-104					
Mean food/day (g)	4.25	4.26	4.25	4.20	4.12**
Total food intake (g)	3092.0	3104.1	3091.4	3056.8	2996.7

**: p < 0.05 in comparison with controls **: p < 0.01 in comparison with controls*

Haematology, clinical chemistry, urinalysis and cholinesterase activity:

Urinalysis and haematological examinations revealed no change. In clinical chemistry, ther was a decrease in cholinesterase activity of the serum, erythrocyte and brain in male and female of 250 ppm or higher dose groups and an increase in glucose in the male1000 ppm group (Table B.6.5.3-3).

Table B.6.5.3-3: 24-month chronic toxicity in mice: Clinical chemistry

Dose level	0 ppm	10 ppm	50 ppm	250 ppm	1000 ppm
Males					
Cholinesterase activity (µmol/ml/min)					
Serum					
Week 52	6.33	6.07	6.05	4.27*** (67%)	2.92*** (46%)
Week 104	8.89	8.89	8.42	6.66*** (75%)	5.06*** (57%)
Erythrocytes					
Week 52	5.22	5.13	5.39	4.28** (82%)	3.67*** (70%)
Week 104	5.21	5.13	5.14	4.54* (87%)	4.53* (87%)
Brain					
Week 52	18.85	18.43	19.00	16.20* (86%)	14.27*** (76%)
Week 104	17.77	17.83	16.59	15.41* (87%)	13.16*** (74%)
Glucose (mg/dl)					
Week 52	117.13	107.50	110.63	105.00	107.51
Week 104	122.27	114.18	127.86	128.29	140.76* (115%)
Females					
Cholinesterase activity (µmol/ml/min)					
Serum					
Week 52	8.51	8.33	7.56	4.83*** (57%)	2.68*** (31%)
Week 104	9.01	9.28	8.58	6.04*** (67%)	3.68*** (41%)
Erythrocytes					
Week 52	5.13	4.97	5.10	3.96* (77%)	3.19*** (62%)
Week 104	5.25	5.08	5.11	4.67* (89%)	4.04*** (77%)
Brain					
Week 52	18.77	17.83	19.86	16.45* (88%)	15.89** (85%)
Week 104	17.92	17.89	18.71	16.61	16.24* (91%)
Glucose (mg/dl)					
Week 52	111.66	109.80	109.92	107.13	103.80
Week 104	122.45	124.98	123.50	122.89	132.14

**: p < 0.05 in comparison with controls*
***: p < 0.01 in comparison with controls*
****: p < 0.001 in comparison with controls*

Gross pathology, organ weights and histopathology:

Ophthalmological examination revealed no change due to the test compound.

An increase in the weight of the pituitary and a decrease of the weight of the thymus was observed in the female 1000 ppm group (Table B.6.5.3-4).

No change considered to be due to the test compound was detected in gross pathological findings at necropsy and histopathological examination. Incidence and severity of neoplasms (Tables B.6.5.3-5 and B.6.5.3-6) were not different between the control and treated groups.

Table B.6.5.3-4: 24-month chronic toxicity in mice: Organ weights

	0 ppm	10 ppm	50 ppm	250 ppm	1000 ppm
Males					
Pituitary					
Absolute (mg)	1.70	1.58	1.54	1.36*	1.76
Relative (%)	4.22	4.12	4.03	3.36*	4.31
Thymus					
Absolute (mg)	20.27	15.10	14.20	14.43	16.63
Relative (%)	48.89	38.78	37.21	34.74	39.91
Females					
Pituitary					
Absolute (mg)	2.13	2.23	2.67	2.09	2.81*
Relative (%)	5.17	5.07	6.42	5.40	7.12*
Thymus					
Absolute (mg)	28.36	25.70	27.07	24.70	23.18*
Relative (%)	68.19	57.29	66.71	63.48	57.76

**: Significantly different from control ($p \leq 0.05$)*

Table B.6.5.3-5: 24-month chronic toxicity in mice: Incidence of malignant and benign neoplasms at week 104

Type of Neoplasms	0 ppm		10 ppm		50 ppm		250 ppm		1000 ppm	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Number of examined animals	50	50	50	50	50	50	50	50	50	50
Malignant neoplasm	32	17(1)	24	28	30	17	30(6)	17	24(4)	22
Benign neoplasm	21	6	21	5	26	8	13	8	15	5

Figures in parentheses represent metastatic neoplasms

Table B.6.5.3-6: 24-month chronic toxicity in mice: Number of animals with neoplasms at week 104

Dose	0 ppm	10 ppm	50 ppm	250 ppm	1000 ppm
Male	36	32	36	32	32
Female	18	23	19	24	22
Total	54/100	55/100	55/100	56/100	54/100

Conclusions

NOAEL: 250 ppm, equivalent to 32.2 mg/kg bw/day - based on cholinesterase changes.

NOEL: 50 ppm, equivalent to 6.4 mg/kg bw/day – based on cholinesterase changes and organ weight changes.

Tolclofos-methyl did not show any carcinogenic activity in this study.

B.6.5.4 Summary and conclusions on long-term toxicity and carcinogenicity

Study	Dose levels	NOEL/NOAEL	Target/main effects
Pence et al., 1982 Chronic toxicity study (2 year) in rats, oral (diet)	0, 100, 300, 1000 ppm (4.2, 12, 42 mg/kg bw/day (males), and 4.8, 15, 49 mg/kg bw/day (females))	**NOAEL** ≥1000 ppm (42 mg/kg bw/day) NOEL ≥1000 ppm (42 mg/kg bw/day)	**Not carcinogenic**
Pence et al., 1985a Chronic toxicity study (2 years) in rats, oral (diet)	0, 100, 300, 1000 ppm (4.1, 12, 42 mg/kg bw/day (males), and 4.8, 15, 49 mg/kg bw/day (females))	**NOAEL** ≥ 1000 ppm (42 mg/kg bw/day) NOEL ≥1000 ppm (42 mg/kg bw/day)	**Not carcinogenic**
Satoh et al., 1983 Chronic toxicity study (2 years) in mice, oral (diet)	0, 10, 50, 250, 1000 ppm (1.3, 6.4, 32.2, 134 mg/kg bw/day (males) and 1.3, 6.9, 34.1, 137 mg/kg bw/day (females))	**NOAEL** 250 ppm (32.2 mg/kg bw/day) NOEL 50 ppm (6.4 mg/kg bw/day)	**↓Cholinesterase levels** ↑ Glucose ↑Pituitary weight ↓Thymus weight **Not carcinogenic**

Tolclofos-methyl did not exhibit evidence of cumulative toxicity in chronic toxicity studies in rats or mice. Some variations in organ weights were noted in the mouse (increased weights of pituitary, and decrease in thymus weight), in the high dose group, but no change was detected in gross pathological findings at necropsy and histopathological examination. In mice, decrease in cholinesterase activity mainly in the serum was seen in the two high dose groups. Changes in the weight of pituitary and thymus were seen in females. There was no effect on general symptoms, time and number of occurrence of external masses and mortality.

In the rat, no distinct compound-related organ or tissue changes were observed in any of the animals. At terminal sacrifice as well as in the animals that died or were sacrificed in extremis during the study, the incidence of interstitial cell tumors was comparable between the compound-treated and control males. Neoplasms, spontaneous disease lesions and incidental findings were consistent with lesions routinely observed in rats of this age and strain. No treatment-related effects were shown on the cholinesterase activity in the 104-week study in rat.

Tolclofos-methyl demonstrated no carcinogenic potential in long-term tests in the rat and mouse. The carcinogenicity data on tolclofos-methyl suggests that it does not present a concern for oncogenicity.

B.6.6 Reproductive toxicity (Annex IIA 5.6)

B.6.6.1 Multi-generation reproductive toxicity studies

B.6.6.1.1 Three generation reproductive toxicity in the rat

Reference	: Pence, D. H., 1985b	**Exposure**	: 15 weeks (pre-mating), 18 days (mating), 3 weeks (gestation), 3 weeks (lactation)
Title of study	: Three-generation reproduction study in rats – S-3349	**Dose**	: 0, 100, 300, 1000 ppm, equivalent to (during 15 weeks pre-mating) in P1, P2 and P3: P1: 0, 6.9, 20.5, 70.6 mg/kg bw/day (males) 0, 8.9, 26.2, 90.5 mg/kg bw/day (females). P2: 0, 7.9, 23.4, 79.6 mg/kg bw/day (males) 0, 9.2, 26.9, 98.5 mg/kg bw/day (females). P3: 0, 7.6, 23.8, 78.2 mg/kg bw/day (males) 0, 9.0, 28.4, 96.1 mg/kg bw/day (females)
Test substance	: Tolclofos-methyl, batch No.: 4, purity: 98.7%, specification No. 01, and batch No.:10901, purity: 97.9 to 98.3%, specification No. 01	**Vehicle**	: Basal diet Purina Rodent Laboratory Chow®
Administration way	: Oral *via* the diet	**GLP statement**	: No, but performed according to GLP of the US Food and Drug administration
Species	: Sprague-Dawley rats	**Guideline**	: In-house method, in accordance with 88/302/EEC , Part B
Group size	: 30/sex/dose (see materials and methods)	**Acceptability**	: Yes
		NOAEL	: ≥1000 ppm (134-198 mg/kg bw/day) (parental, reproductive and pup)

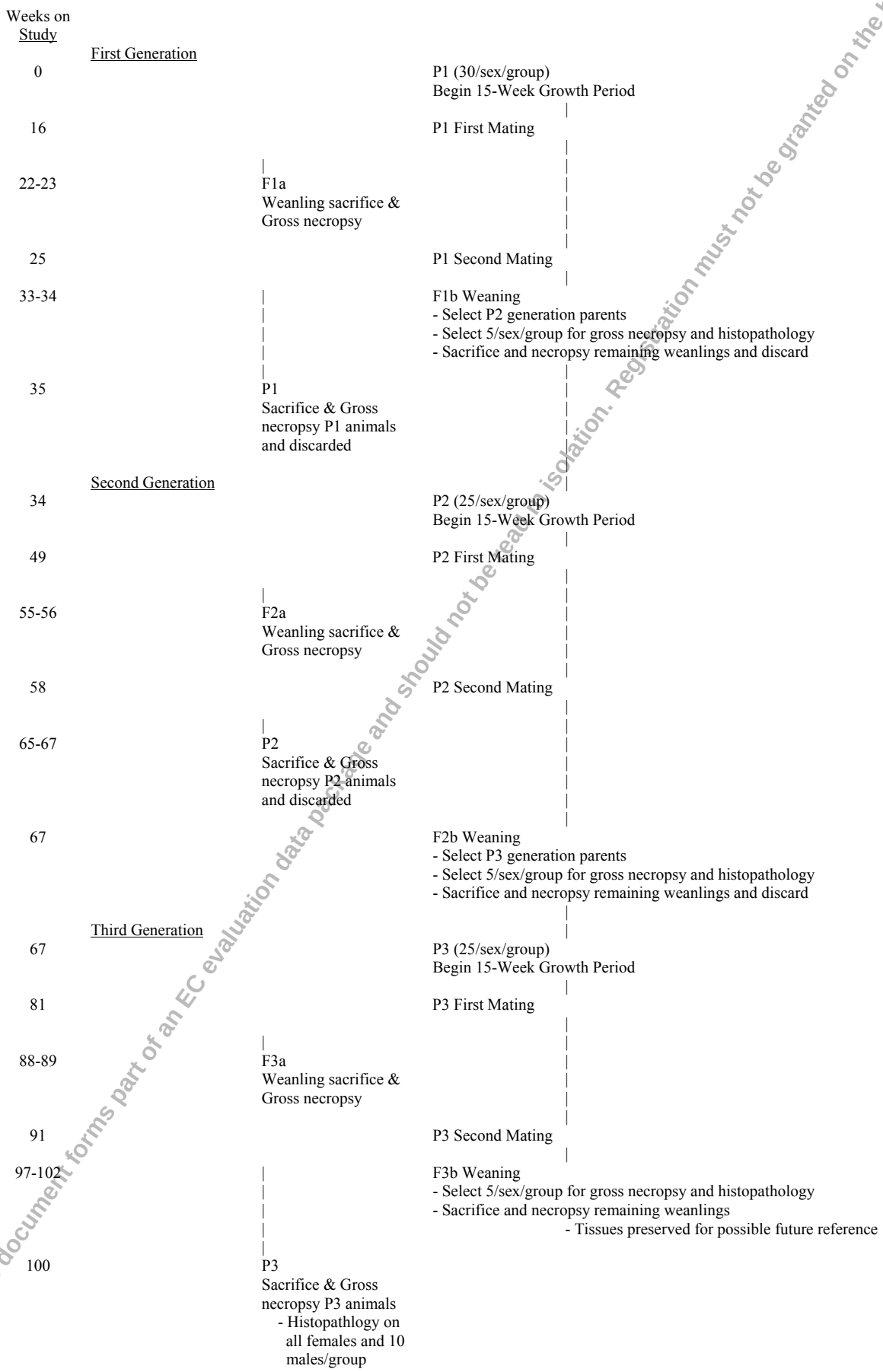
Materials and methods

The day on which evidence of mating was observed was designated as Day 0 of gestation.

On Day 4 of lactation, the number of F1 pups was reduced to a maximum number of 10 per litter (5 males and 5 females, when possible). All offspring from the first mating of each generation (F1a, F2a and F3a) were necropsied and discarded after the 21-day lactation period. Offspring from the second matings of the P1 and P2 generations (F1b and F2b) were maintained through weaning, then 25 animals/sex/group were selected as parental animals for the succeeding generation. Five weanling animals/sex/group from the F1b, F2b and F3b generations were selected for gross necropsy and histopathologic examination. The F3b weanlings not selected were necropsied and tissues were preserved for possible future examination. The remaining pups from F1b and F2b generations were necropsied and discarded. After the second litter of each generation completed weaning,

all surviving parental males and females were necropsied. Diagrammatic outline of the three generations reproduction study is shown in Figure B.6.6.1.1-1. According to guidelines, only two generations are needed.

Figure B.6.6.1.1-1: Diagrammatic outline of the three generation reproduction study in rats



Findings

1. Parental data

Mortality and clinical signs:

Mortatlities were evenly distributed among dose groups. The deaths and moribund sacrifices were considered incidental and not related to compound administration. None of the clinical signs observed was considered to be compound-related.

Body weights and food consumption:

During the 15-week growth periods, parental body weights, growth rates, and total food consumption were comparable between treated and control groups. Mean maternal body weights and body weight changes during gestation and lactation generally were comparable among groups of each generation. The mean body weights of the high-dose P2 males, and the mid- and high-dose P3 males recorded at four-week intervals after mating were lower than respective control weights, but growth rates were similar to the respective controls.

Gross pathology:

There were no consistent gross pathology findings for any of the parental rats that could be attributed to administration of the compound.

Organ weights and organ/body weight ratios:

No compound related effects on organ weights were observed.

Histopathology:

No compound related effects with respect to histopathology were observed.

Reproduction indices:

Pregnancy rates, fertility rates and parturition indices were generally comparable among groups of each generation (Tables 6.6.1.1-4A to 4F).

2. Offspring data

Survival indices and growth (F1a, F2a, F3a, F1b, F2b, F3b):

There were no apparent differences in any of the offspring viability and survival data (Tables B.6.6.1.1-4A to 4F). There was a tendency towards increased percentages of male pups per litter during the study. The connection to treatment is obscure, since the values fell within the normal range of the laboratory.
No significant reproductive effects were noted during the study.

Clinical observations:

No compound-related clinical signs were noted for any of the offspring (F1a, F2a, F3a, F1b, F2b, F3b).

Gross pathology:

Gross pathology findings for the offspring of all generations were considered incidental in nature and showed no relation to compound administration.

Organ weights and organ/body weight ratios (F1b, F2b, F3b):

Evaluation of the organ weight data revealed no consistent trends that could be attributed to the treatment

Histopathology (F1b, F2b, F3b):

Microscopic evaluation did not reveal any compound-related histomorphologic alterations in the tissues examined. A variety of spontaneous disease lesions and incidental findings were noted, within the usual background data of this strain of rats.

Table B.6.6.1.1-4A: Three generation reproduction in rats: Maternal reproduction indices and offspring survival and growth (P1F1a)

Dose level	0 ppm	100 ppm	300 ppm	1000 ppm
Parental reproduction indices				
Total No. of females placed with males	30	30	30	29
Total No. of females showing evidence of mating	26	30	26	27
Total No. of females pregnant	20	24	17	20
Mating index (%)	86.7	100.0	86.7	93.1
Pregnancy rate (%)	66.7	80.0	56.7	69.0
Total No. of males placed with females	29	30	30	29
Total No. of males mated with females	22	26	23	21
Male fertility rate (%)	75.9	86.7	76.7	72.4
No. of females delivering offspring	20	24	17	20
No. of females delivering viable offspring	20	24	17	20
Parturition index (%)	100.0	100.0	100.0	100.0
Offspring indices				
Mean No. of live pups at parturition/litter	11.6	12.2	11.6	11.9
Live birth index (%)	98.2	98.5	96.5	98.7
Neonatal survival index (%)	90.5	96.4	93.7	86.8
Survival at Day 7 (%)	98.3	99.6	97.3	99.4
Survival at Day 14 (%)	97.7	96.5	96.4	97.8
Weaning survival index (%)	97.7	96.0	96.4	97.2
Percent males at Day 21	51.9	50.1	47.4	57.0
Mean offspring body weights (g)				
Males at Day 21	34.8	36.4	37.9	33.4
Females at Day 21	34.5	34.5	35.9	32.1

Parental Reproduction indices
Mating index (%) = (Number of females showing evidence of mating/number of females with males) x 100
Pregnancy rate (%) = (Number of females pregnant or showing evidence of parturition/number of females placed with males) x 100
Male fertility rate (%) = (Number of males mated with one or more females/number of males placed with females) x 100
Parturition index (%) = (Number of females delivering viable offspring/total number of females with litters) x 100
(Note: Number of males mated with females = evidence of sperm or vaginal plug in female, or confirmed pregnancy. In cases of multiple matings of males with a single female and subsequent conception, the last male placed with the females was considered to be the sire.)
Offspring indices
Live birth index (%) = Group mean of (per litter number of pups alive/total pups observed at Day 1) x 100
Neonatal survival index (%) = Group mean of (per litter number of pups alive at Day 4 precull/total pups observed at Day 1) x 100
Weaning survival index (%) = Group mean of (per litter number of pups alive at Day 21/total pups not culled at Day 4) x 100
Percent males = (Number of male pups/total number of pups) x 100

Table B.6.6.1.1-4B: Three generation reproduction in rats: Maternal reproduction indices and offspring survival and growth (P1F1b)

Dose level	0 ppm	100 ppm	300 ppm	1000 ppm
Parental reproduction indices				
Total No. of females placed with males	29	30	29	29
Total No. of females showing evidence of mating	28	29	27	29
Total No. of females pregnant	17	20	15	23
Mating index (%)	96.6	96.7	93.1	100
Pregnancy rate (%)	58.6	66.7	51.7	79.3
Total No. of males placed with females	29	30	29	29
Total No. of males mated with females	23[a]	26[a]	25	25[a]
Male fertility rate (%)	79.3	86.7	86.2	86.2
No. of females delivering offspring	16	20	15	22
No. of females delivering viable offspring	16	20	15	22
Parturition index (%)	100.0	100.0	100.0	100.0
Offspring indices				
Mean No. of live pups at parturition/litter	12.5	11.7	11.7	11.6
Live birth index (%)	98.4	96.4	98.1	99.4
Neonatal survival index (%)	95.3	99.2	99.5	99.0
Survival at Day 7 (%)	100.0	99.0	99.3	99.5
Survival at Day 14 (%)	100.0	98.5	98.0	98.6
Weaning survival index (%)	99.4	98.5	98.0	97.7
Percent males at Day 21	47.0	51.3	48.4	53.7
Mean offspring body weights (g)				
Males at Day 21	38.4	38.2	41.7	38.1
Females at Day 21	36.8	36.3	40.2	35.4

[a]: *Includes mating to one female which subsequently died*
Other footnotes see Table B.6.6.1.1-4A

Table B.6.6.1.1-4C: Three generation reproduction in rats: Maternal reproduction indices and offspring survival and growth (P2F2a)

Dose level	0 ppm[a]	100 ppm	300 ppm	1000 ppm
Parental reproduction indices				
Total No. of females placed with males	24	25	25	25
Total No. of females showing evidence of mating	23	25	25	24
Total No. of females pregnant	22	22	18	22
Mating index (%)	95.8	100.0	100.0	96.0
Pregnancy rate (%)	92	88.0	72.0	88.0
Total No. of males placed with females	24	25	25	25
Total No. of males mated with females	22	23	23	24
Male fertility rate (%)	91.7	92.0	92.0	96.0
No. of females delivering offspring	22	22	18	22
No. of females delivering viable offspring	22	22	18	22
Parturition index (%)	100.0	100.0	100.0	100.0
Offspring indices				
Mean No. of live pups at parturition/litter	11.9	11.1	9.4	12.5
Live birth index (%)	97.8	96.1	95.3	98.0
Neonatal survival index (%)	99.1	94.6	98.7	96.9
Survival at Day 7 (%)	99.0	98.4	97.6	97.1
Survival at Day 14 (%)	98.6	96.9	97.6	96.6
Weaning survival index (%)	98.0	96.9	97.6	95.3
Percent males at Day 21	44.1	53.6	51.6	51.2
Mean offspring body weights (g)				
Males at Day 21	37.0	38.1	38.0	35.4
Females at Day 21	36.1	36.8	36.2	34.1

[a]: *All litter information from female no 15799 was excluded from offspring indices due to female being mated with a litter mate*
Other footnotes see Table B.6.6.1.1-4A

Table B.6.6.1.1-4D: Three generation reproduction in rats: Maternal reproduction indices and offspring survival and growth (P2F2b)

Dose level	0 ppm	100 ppm	300 ppm	1000 ppm
Parental reproduction indices				
Total No. of females placed with males	24	25	24	25
Total No. of females showing evidence of mating	23	25	22	23
Total No. of females pregnant	17	17	15	20
Mating index (%)	95.8	100.0	91.7	92.0
Pregnancy rate (%)	70.8	68.0	62.5	80.0
Total No. of males placed with females	24	25	25	24
Total No. of males mated with females	18	24	15	20
Male fertility rate (%)	75.0	96	60	83.3
No. of females delivering offspring	16	17	13	20
No. of females delivering viable offspring	16	17	13	19
Parturition index (%)	100.0	100.0	100.0	95.0
Offspring indices				
Mean No. of live pups at parturition/litter	11.2	10.5	9.6	12.7
Live birth index (%)	95.6	99.5	98.3	94.7
Neonatal survival index (%)	97.9	100.0	99.4	98.9
Survival at Day 7 (%)	100.0	99.4	100.0	99.5
Survival at Day 14 (%)	98.8	98.8	99.2	99.5
Weaning survival index (%)	97.5	98.8	99.2	98.9
Percent males at Day 21	52.5	49.6	54.7	45.0
Mean offspring body weights (g)				
Males at Day 21	38.0	38.1	35.9	37.4
Females at Day 21	36.0	36.3	35.7	34.4

Footnotes see Table B.6.6.1.1-4A

Table B.6.6.1.1-4E: Three generation reproduction in rats: Maternal reproduction indices and offspring survival and growth (P3F3a)

Dose level	0 ppm	100 ppm	300 ppm	1000 ppm
Parental reproduction indices				
Total No. of females placed with males	25	25	25	25
Total No. of females showing evidence of mating	24	24	23	23
Total No. of females pregnant	20	18	17	20
Mating index (%)	96.0	96.0	92.0	92.0
Pregnancy rate (%)	80.0	72.0	68.0	80.0
Total No. of males placed with females	24	25	25	25
Total No. of males mated with females	16	22	21	21
Male fertility rate (%)	66.7	88.0	84.0	84.0
No. of females delivering offspring	20	18	17	20
No. of females delivering viable offspring	20	18	17	20
Parturition index (%)	100.0	100.0	100.0	100.0
Offspring indices				
Mean No. of live pups at parturition/litter	11.7	11.4	12.0	11.7
Live birth index (%)	98.9	98.1	99.1	98.9
Neonatal survival index (%)	97.2	92.8	98.7	98.6
Survival at Day 7 (%)	99.5	100.0	98.8	100.0
Survival at Day 14 (%)	99.5	93.4	98.8	100.0
Weaning survival index (%)	99.0	93.4	98.2	100.0
Percent males at Day 21	49.7	46.1	49.8	46.6
Mean offspring body weights (g)				
Males at Day 21[a]	37.2	36.1	36.4	35.3
Females at Day 21	35.5	33.6	34.8	33.7

[a]: *Log_{10} transformed data analysed*
Other footnotes see Table B.6.6.1.1-4A

Table B.6.6.1.1-4F: Three generation reproduction in rats: Maternal reproduction indices and offspring survival and growth (P3F3b)

Dose level	0 ppm	100 ppm	300 ppm	1000 ppm
Parental reproduction indices				
Total No. of females placed with males	24	24	24	25
Total No. of females showing evidence of mating	20	22	22	23
Total No. of females pregnant	16	17[a]	18	20
Mating index (%)	83.3	91.7	91.7	92.0
Pregnancy rate (%)	67.0	70.1	75.0	80.0
Total No. of males placed with females	24	24	24	25
Total No. of males mated with females	19	21	21	22
Male fertility rate (%)	79.2	87.5	87.5	88.0
No. of females delivering offspring	16	16	18	20
No. of females delivering viable offspring	16	16	18	20
Parturition index (%)	100.0	100.0	100.0	100.0
Offspring indices				
Mean No. of live pups at parturition/litter	11.8	13.0	10.7	12.2
Live birth index (%)	98.3	96.6	94.7	96.8
Neonatal survival index (%)	100.0	99.4	89.2	93.3
Survival at Day 7 (%)	98.8	99.4	98.8	98.5
Survival at Day 14 (%)	95.6	93.8	81.5	96.9
Weaning survival index (%)	94.9	93.1	80.8	95.8
Percent males at Day 21	48.4	49.9	50.6	56.0
Mean offspring body weights (g)				
Males at Day 21	41.5	38.0	38.0	38.0
Females at Day 21[b]	39.3	36.7	35.3	35.9

[a]: *Includes one found dead female which was pregnant*
[b]: *Log_{10} transformed data analyzed*
Other footnotes see Table B.6.6.1.1-4A

Conclusions

There were no reproductive effects under the dose regimens and conditions used in this study. However, the dose levels can be considered to be low and do not give rise to apparent maternal toxic effect. Despite of that, tolclofos-methyl does not seem to lead to harmful reproductive effects.

The following NOAELs and NOELS were concluded from the study:

Parental NOAEL/NOEL: ≥1000 ppm (70.6-98.5 mg/kg bw/day).

Pup NOEL/NOAEL: ≥1000 ppm.

Reproductive NOAEL/NOEL: ≥1000 ppm.

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B.6.6.1.2 Dominant lethal assay for male fertility: Rat dominant lethal assay

The study was also performed to evaluate genotoxicity and is presented at point B.6.4.2.3

B.6.6.2 Developmental toxicity studies

B.6.6.2.1 Teratology study in rats (1st study)

Reference	: Pence, D. H., 1979a	**Exposure**	: Days 6 – 15 during gestation
Title of study	: Teratology study in rats – S-3349	**Dose**	: 0, 5, 15, 50 mg/kg bw/day
Test substance	: Tolclofos-methyl, batch No.: 0523, purity: 94.9%, specification No. 02	**Vehicle**	: Methylcellulose (1 ml/kg bw)
Administration way	: Oral *via* gavage	**GLP statement**	: No, but performed according to GLP of the US Food and Drug administration
Species	: Fischer 344 CD®F rats	**Guideline**	: In-house method, equivalent to OECD 414 and 88/302/EEC, part B
Group size	: 30/dose	**Acceptability**	: Yes
		NOAEL	: ≥50 mg/kg bw/day (maternal and developmental toxicity)

Materials and methods

Tolclofos-methyl was administered daily from days 6 to 15 of gestation. The day vaginal plugs and/or sperm were observed was designated as day 0 of gestation. On day 19 of gestation, all dams were killed for fœtus sampling and examination.

A pilot dose toleration study was conducted first to determine if the high dose level of 50 mg/kg bw/day is well tolerated: 3 females were treated at 50 mg/kg/day for three consecutive days.

Findings

1. Pilot study

The three female rats dosed at 50 mg/kg bw/day for three consecutive days were able to tolerate that level, and it was, therefore, subsequently used as the high dose in the teratology study.

2. Maternal data

General observations:

No maternal deaths occurred during the study (including those females which were placed on study but were found not to be pregnant). A comparable incidence of clinical signs was noted between the control and treated groups. Mean body weight changes and mean food consumption values were comparable between the control and treated groups.

Gravid uterus weights:

Comparable uterus weights were noted between the control and treated groups.

Gross pathology:

The incidence of findings were comparable between dose groups.

3. Caesarean data (Table B.6.6.2.1-1)

Pregnancy rates:

Higher pregnancy rates were noted in the treated groups than in the control group.

Implantations and Corpora lutea:

The mean implantation efficiency (number of implantations/number of corpora lutea) of the treated groups were noted to be lower than the value of the control group, with a statistically significantly lower value in the high-dose group.

Foetal viability, sex and weight:

The incidence of fœtal death was comparable between the control and treated groups. Mean fœtal weights and mean fœtal lengths were comparable between the dose groups and control. Other parameters were also similar between the groups.

Table B.6.6.2.1-1: Teratogenicity in rats: Caesarean data

Dose level	0 mg/kg/day	5 mg/kg/day	15 mg/kg/day	50 mg/kg/day
Number of females mated	30	30	30	30
Number of rats pregnant	21	23	26	26
Pregnancy rate (%)	70.0	76.7	86.7	86.7
Mean number of				
Corpora lutea	11.0	11.7	11.3	11.2
Implantations	10.1	9.6	9.6	9.6
Resorptions	1.0	0.7	0.8	0.4
Fetuses -Dead	0	0.1	0	0.04
-Alive	9.1	8.9	8.8	9.2
Indices calculated on per litter basis				
Mean implantation efficiency (%)	91.9	83.3	85.5	86.1*
Mean incidence of resorption (%)	10.0	7.0	9.2	4.4
Mean incidence of fetal death (%)	0	1.0	0	0.4
Mean incidence of fetal viability (%)	90.0	92.1	90.8	95.2
Live male fetuses				
Mean body weight (g)	2.21	2.21	2.20	2.22
Mean crown-rump distance (cm)	3.21	3.18	3.20	3.18
Live female fetuses				
Mean body weight (g)	2.11	2.10	2.11	2.14
Mean crown-rump distance (cm)	3.15	3.11	3.15	3.12
Mean sex ratio	1.17	1.15	1.83	1.40
Mean uterine weight (g)	36.13	34.99	34.87	37.38

**: Significantly different from control ($p \leq 0.05$)*

4. Fœtal evaluations (Table B.6.6.2.1-2)

External and visceral evaluations:

No diffences in the incidence of external and visceral malformations were noted.

Skeletal evaluations:

Skeletal examination revealed no anomalies. Angulated ribs were noted in one 50 mg/kg bw/day fœtus, and lagging ossification of the sternebrae, pubis and/or caudal vertebrae was noted at slightly higher frequencies in the treated groups than in the control group. However, the findings were not dose related nor ststistically significant.

Table B.6.6.2.1-2: Teratogenicity in rats: Foetal visceral and skeletal variations

Dose level	0 mg/kg bw/day	5 mg/kg bw/day	15 mg/kg bw/day	50 mg/kg bw/day
Total number of litters examined[a]	21	23	26	26
Mean number of				
Visceral anomalies	0	0	0	0
Visceral variants	0	0	0	0.04
Skeletal anomalies	0	0	0	0
Skeletal variants	0.5	1.1	0.9	0.8
Indices calculated on per litter basis				
Mean incidence of visceral anomalies (%)	0	0	0	0
Mean incidence of visceral variants (%)	0	0	0	1.3
Mean incidence of skeletal anomalies (%)	0	0	0	0
Mean incidence of skeletal variants (%)	7.5	17.3	13.1	12.2

a: Only data from live pups were used in calculations of means.

Conclusions Tolclofos-methyl does not possess any teratogenic activity under the dose regimens used in this study.

NOAEL: ≥50 mg/kg bw/day (maternal and developmental toxicity).

NOEL: 15 mg/kg bw/day – based on decreased mean implantation efficiency in the 50 mg/kg bw/day dose group. This difference is, however, considered incidental, since in the next study (B.6.6.2.2), there is no such difference between control and the 1000 mg/kg bw/day dose group.

B.6.6.2.2 Teratology study in rats (2nd study)

Reference	: Morseth, S. L., 1987	**Exposure**	: Days 6 – 15 of gestation
Title of study	: Teratolgy study of S-3349 T.G. in rats	**Dose**	: 0, 100, 300, 1000 mg/kg bw/day
Test substance	: Tolclofos-methyl, batch No.: 40807, purity: 96.7%, specification No. 01	**Vehicle**	: Methylcellulose (5 ml/kg bw)
Administration way	: Oral *via* gavage	**GLP statement**	: No (quality assurance review according to GLP)
Species	: Sprague-Dawley rats	**Guideline**	: In-house method, equivalent to OECD 414 and 88/302/EEC, Part B
Group size	: 23/dose	**Acceptability**	: Yes
		NOAEL	: ≥1000 mg/kg bw/day (maternal toxicity and developmental toxicity)
		NOEL	: 300 mg/kg bw/day (maternal and developmental toxicity)

Materials and methods

Tolclofos-methyl was administered daily from Days 6 to 15 of gestation. The day vaginal plugs and/or sperm were observed was designated at Day 0 of gestation. On Day 20 of gestation, all dams were killed for fœtus sampling and examination.

Findings

1. Maternal data

General observations:

All females survived to Day 20 of presumed gestation and no clinical sign was related to the treatment. Mean body weight gains were significantly less than control values for Days 6-11 (groups 100 and 1000 mg/kg bw/day). The mean net body weight gain values (Days 0-20) showed a negative trend at 1000 mg/kg bw/day than the corresponding control values (Table B.6.6.2.2-1).

Gravid uterus weights and carcass weights:

No treatment-related differences were noted in the treated groups when compared to the control group.

Gross pathology:

The findings were considered to be incidental and comparable among dose groups.

Table B.6.6.2.2-1: Teratogenicity in rats: Body weights (g)

Dose level	0 mg/kg/day	100 mg/kg/day	300 mg/kg/day	1000 mg/kg/day
Body weight				
Day 20	366	360	363	362
Gravid Uterine Weight	79.9	76.7	78.6	80.9
Carcass Weight	286.3	282.7	284.3	281.2
Body weight gain				
Day 0-6	34	35	34	32
Day 6-7	0	0	-2	-1
Day 6-11	18	16*	16	13*
Day 6-20	107	103	105	102
Day 16-20	57	58	56	57
Day 0-20	141	137	138	134
Net Body Weight Change[a]	61.6	60.4	59.2	52.9

**: Significantly different from control ($p \leq 0.05$)*
a: significant negative trend ($p \leq 0.05$)

2. Caesarean data (Table 6.6.2.2-3)

Survival and pregnancy rates:

The maternal survival rates were 100% at Day 20 caesarean section for all groups. The pregnancy rates were 83, 96, 100 and 96% for groups 0, 100, 300 and 1000 mg/kg bw/day, respectively. No signs of premature delivery were observed.

Implantations and Corpora lutea:

A comparison of the mean number of implantation and corpora lutea revealed no treatment-related differences. Statistical analysis of percent implantation efficiency revealed no significant differences between control and treated groups.

Foetal viability, sex and weight:

Statistical evaluation of fœtal weights, percent males per litter and percent resorptions revealed no significant differences between control and treated groups.

Table B.6.6.2.2-3: Teratogenicity in rats: Caesarean data

Dose level (mg/kg bw/day)	0	100	300	1000
Number (%) of:				
Females mated	23	23	23	23
Females surviving to Day 20 cesarean section	23 (100)	23 (100)	23 (100)	23 (100)
Females pregnant	19 (83)	22 (96)	23 (100)	22 (96)
Pregnant females surviving to Day 20 cesarean section	19 (100)	22 (100)	23 (100)	22 (100)
Females delivering early	0 (0)	0 (0)	0 (0)	0 (0)
Litters examined	19	22	23	22
Mean number (%) of :				
Corpora lutea	17.6[a]	16.7	16.9	17.1
Implantations	15.3	15.0	14.8	15.8
Live fetuses	14.8 (97)	14.2 (95)	14.3 (97)	15.0 (95)
Dead fetuses	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Early resorptions	0.5 (3)	0.7 (5)	0.5 (3)	0.8 (5)
Late resorptions	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Total resorptions	0.5 (3)	0.7 (5)	0.5 (3)	0.8 (5)
Male fetuses	7.7 (52)	7.4 (52)	7.3 (51)	7.7 (52)
Mean:				
Implantation efficiency (%)	90[a]	90	89	93
Fetal weight (g)	3.3	3.5	3.5	3.4
Male	3.4	3.5	3.6	3.4
Female	3.3	3.4	3.4	3.3
Covariate adjusted mean:				
Fetal weight (g)	3.3	3.5	3.5	3.4
Male	3.4	3.5	3.6	3.4
Female	3.3	3.4	3.4	3.3

a: Based on 18 litters; value for one animal was excluded.

3. Fœtal evaluations

There was a comparable incidence of external malformations and visceral variations between dose groups. From the skeletal evaluations it was found that the number of foetuses with the 5th and/or 6th sternebrae unossified fluctuated and was significantly greater at 1000 mg/kg/day than in the control group; this delay in ossification was considered to be treatment-related. Other variations in development occurred in a non-dose-related pattern. Neither the type nor frequency of malformations indicated a treatment-related response.

Table B.6.6.2.2-4: Teratogenicity in rats: Foetal skeletal variations

Dose level (mg/kg bw/day)		0	100	300	1000
Litters evaluated	No.	19	22	23	22
Fetuses evaluated	No.	139	154	163	168
Live	No.	139	154	163	168
Dead	No.	0	0	0	0
5th sternebra unossified					
Fetal incidence[T]	No.	41	46	52	75*
	%	29	30	32	45
Litter incidence	No.	14	14	13	18
	%	74	64	57	82
6th sternebra unossified					
Fetal incidence	No.	5	12	4	19*
	%	3.6	7.8	2.5	11
Litter incidence	No.	4	6	3	8
	%	21	27	13	36

**: Significantly different from control (p≤ 0.05)*
[T]: Significant positive trend, (p≤ 0.05)No.: Number %: Percent

Conclusions

The findings indicate a slight effect of tolclofos-methyl at 1000 mg/kg bw/day, giving the following NOAEL/NOELs:
NOAEL (maternal toxicity): ≥1000 mg/kg bw/day
NOEL (maternal toxicity): 300 mg/kg bw/day - based on the significant negative trend on body weight gain at 1000 mg/kg bw/day.

NOAEL (developmental toxicity): ≥1000 mg/kg bw/day
NOEL (developmental toxicity): 300 mg/kg - based on the the fact that the number of foetuses with 5th and/or 6th sternebrae unossified was significantly greater at 1000 mg/kg bw/day. However, the unossification of the 5th sternebae was considered to be a developmental variation and in the variation of the 6th sternebrae there was no dose-response.

B. 6.6.2.3 Teratology study in rabbits

Reference	: Kashima, M., 1991	**Exposure**	: Days 6 – 18 of gestation
Title of study	: Teratology study of S-3349 in rabbits (Study performed 1981)	**Dose**	: 0, 300, 1000, 3000 mg/kg bw/day
Test substance	: Tolclofos-methyl, batch No.: 4, purity: 98.7%, specification No. 01	**Vehicle**	: Carboxymethylcellulose (5 ml/kg bw)
Administration way	: Oral *via* gavage	**GLP statement**	: No (Quality assurance review performed later according to GLP)
Species	: New Zealand White rabbits	**Guideline**	: In-house method equivalent to OECD 414 and 88/302/EEC
Group size	: See materials and methods	**Acceptability**	: Yes
		NOAEL	: 300 mg/kg bw/day (maternal toxicity), ≥3000 mg/kg bw/day (developmental toxicity)
		NOEL	: < 300 mg/kg bw/day (maternal toxicity)

Materials and methods

Tolclofos-methyl was administered daily from Days 6 to 18 of gestation. The day when sperms were found in the vaginal smear was designated as Day 0 of gestation. On Day 29 of gestation, all dams were killed for fœtus sampling and examination.

The number of pregnant females at each dose level was as follows:

Dose level	0 mg/kg bw/day	300 mg/kg bw/day	1000 mg/kg bw/day	3000 mg/kg bw/day
No of animals	15	14	13	17

Findings

1. Maternal data

General observations:

There were no remarkable clinical findings, but one dam in the 3000 mg/kg/day group showed suppression or decreases in body weight gain from Day 8 of gestation and decreases in food consumption from Day 9 of gestation and died on Day 14 of gestation. No maternal death occurred in the other groups. Abortion was found in one dam on day 26 of gestation in the 1000 mg/kg bw/day group and in two dams on day 20 and 22, resoectively, in the 3000 mg/kg bw/day group. The body weight gains and food consumption were suppressed in the 1000 mg/kg bw/day group or above (Tables B.6.6.2.3-1 and -2), showing slight maternal toxic effect. The food consumption was markedly lower in the dams in which the three abortions occurred, even when compared with dams in the same dosage group. This shows a probable connection between maternal toxicity and the abortions.

The significantly decreased maternal body weights observed in all treated groups, may be attributable to lower mean body weight already at commencement of treatment.

Table B.6.6.2.3-1: Teratogenicity in rabbits: Body weight and body weight gain

Dose level	0 mg/kg/day	300 mg/kg/day	1000 mg/kg/day	3000 mg/kg/day
Body weight (kg)				
Day 0	3.00	2.71*	2.64**	2.75*
Day29	3.68	3.32*	3.26*	3.29*
Body weight gain (kg)				
Day 0-29	0.68	0.62	0.65	0.55
Day 6-19	0.34	0.27	0.15*	0.08**

**: Significantly different from control ($p \leq 0.05$)*
***: Significantly different from control ($p \leq 0.01$)*

Table B.6.6.2.3-2 Teratogenicity in rabbits: Food consumption

Dose level	0 mg/kg bw/day	300 mg/kg bw/day	1000 mg/kg bw/day	3000 mg/kg bw/day
Day 0-6 (Average)[b]	151.25	136.07	144.82	142.85
Day 7	155.20	125.71	111.38**	136.12
Day 8	155.87	138.57	117.08*	120.12**
Day 9	147.87	139.43	99.54**	108.94**
Day 10	163.73	150.86	110.00***	128.35*
Day 11	166.80	160.71	135.85*	131.53*
Day 12	155.60	137.00	123.08**	111.88**
Day 13	149.33	136.71	136.15	111.29*
Day 14	144.80	132.57	128.15	114.50*
Day 15	146.13	127.00	124.92	111.00*
Day 16	139.47	116.14	125.54	124.88
Day 17	142.40	123.43	118.62	125.75
Day 18	141.60	134.29	120.00	125.75
Day 19	138.27	120.43	112.08	125.00
Day 28-29 (Total of 2 days)[c]	254.00	231.86	220.83	251.71
Day 28-29 (Average)[b]	127.00	115.93	110.42	125.86

[a]: g/day [c]: g/2 days
**: Significantly different from control ($p \leq 0.05$)*
***: Significantly different from control ($p \leq 0.01$)*
****: Significantly different from control ($p \leq 0.001$)*

Necropsy findings and maternal organ weights:

No grossly abnormal changes were observed in any dams including one dead dam. At doses of 1000 mg/kg bw/day and above, there was a significant decrease in the weight of kidneys and spleen (Table B.6.2.2.3-3). The

significant decreases in the liver and urinary bladder weights were not dose-related and and the relationship to treatment is obscure.

Table B.6.6.2.3-3: Organ weigts of female rabbits treated orally with tolclofos-methyl

Week 4	0 mg/kg bw/day	300 mg/kg bw/day	1000 mg/kg bw/day	3000 mg/kg bw/day
Body weight (kg)	3.68	3.32*	3.26*	3.29*
Kidney (Right) (g)	9.19	8.60	8.50	8.20*
Kidney (Left) (g)	9.35	8.61	8.22*	8.18*
Spleen (g)	1.47	1.52	1.76	1.17*
Liver (g)	104.54	92.05*	94.87	106.91
Urinary bladder (g)	1.84	1.47*	1.45*	1.63

**: Significantly different from control ($p < 0.05$)*
N=15

2. Caesarean data (Table B.6.6.2.3-4)

There were no significant differences in the data between the control and treated groups.

Table B.6.6.2.3-4: Teratogenicity in rabbits: Caesarean data

Dose level	0 mg/kg bw/day	300 mg/kg bw/day	1000 mg/kg bw/day	3000 mg/kg bw/day
Number of animals	15	14	12	14
No. of implantations				
Total	124	121	102	109
Mean	8.27	8.64	8.50	7.79
No. of live fetuses				
Total (%)	113 (91.1%)	109 (90.1)	94 (92.2)	89 (81.7)
Mean	7.53	7.79	7.83	6.36
No. of dead fetuses				
Total	0	0	0	0
%	0	0	0	0
Resorbed embryo				
Total (%)	11 (8.9)	12 (9.9)	8 (7.8)	20 (18.3)
Implantation trace				
Total (%)	0	0	0	8 (7.3%)
Placental remnant				
Total (%)	3 (2.4)	4 (3.3)	3 (2.9)	4 (3.7)
Early resorption				
Total (%)	3 (2.4)	3 (2.5)	3 (2.9)	5 (4.6)
Late resorption				
Total (%)	4 (3.2)	4 (3.3)	1 (1)	2 (1.8)
Macerate fetus				
Total (%)	1 (0.8)	1 (0.8)	1 (1)	1 (0.9)
Immature infant				
Total	10	27	7	13
%	8.8	24.8	7.4	14.6
Malformation				
Total	0	0	0	0
Fetus body weight (g)				
Male				
Number	13	14	11	12
Mean	42.38	37.27	38.93	38.62
Female				
Number	15	13	12	13
Mean	38.14	34.45	36.40	36.26
Sex ratio				
Male				
Number	13	14	11	12
Total (%)	58 (51.3)	64 (58.7)	45 (47.9)	46 (51.7)
Mean	4.46	4.57	4.09	3.83
Female				
Number	15	13	12	13
Total (%)	55 (48.7)	45 (41.3)	49 (52.1)	43 (48.3)
Mean	3.67	3.46	4.08	3.31

3. Fœtal evaluations

Neither external nor visceral evaluations revealed any apparent differences between dose groups.

In the skeletal evaluations, skeletal variations included eight lumbar vertebrae and 13th rib or asymmetry of sternebrae, but the incidences of these variations were not significantly increased and there were no dose-response. Neither the type nor frequency of malformations indicated an embryo-toxic or teratogenic response. There were no effects on the degree of ossification.

Conclusions

NOAEL (maternal toxicity): 300 mg/kg bw/day – based on death of one dam in the highest dose and decreased body weight gain and food consumption in the two highest doses.

NOEL (maternal toxicity): < 300 mg/kg bw/day – based on decrease of body weight and slightly reduced kidney and spleen weight at the two highest dose levels.

NOAEL/NOEL (developmental toxicity): ≥3000 mg/kg bw/day

Comment from RMS: It can be difficult to conclude if abortions are coupled to maternal toxicity or reflects a developmental defect. However, food consumption was, in this study, markedly lower in the dams in which the three abortions occurred. This shows a probable connection between maternal toxicity and the abortions.

Furthemore, the highest dose (3000 mg/kg bw/day) is unnecessary high and is higher than the recommended highest dose in the guidelines.

B.6.6.3 Summary and conclusions on reproductive toxicity

Study	Dose levels	NOEL/NOAEL	Targets/main effects
Pence et al., 1985b 3 generation reproduction study in rats, oral	0, 100, 300, 1000 ppm males/females: 6.9-7.9, 20.5-23.8, 70.6-79.6 / 8.9-9.2, 26.2-28.4, 90.5-98.5 mg/kg bw/day	**Parental NOAEL/NOEL** ≥1000 ppm (70.6-98.5 mg/kg bw/day). Pup **NOEL** ≥1000 ppm Reproduction **NOEL** ≥1000 ppm	
Pence et al., 1979 Teratology study in rats, oral	0, 5, 15, 50 mg/kg bw/day	**Maternal and developmental NOAEL** ≥50 mg/kg bw/day **NOEL** 15 mg/kg bw/day	↓ Implamantation efficiency
Morseth et al., 1987 Teratology study in rats, oral	0, 100, 300, 1000 mg/kg bw/day	**Maternal NOAEL** ≥1000 mg/kg bw/day NOEL 300 mg/kg bw/day **Developmental NOAEL** ≥1000 mg/kg bw/day NOEL 300 mg/kg bw/day Not teratogenic	↓ Body weight gain Delayed ossification Not teratogenic

Study	Dose levels	NOEL/NOAEL	Targets/main effects
Kashima et al., 1991 Teratology study in rabbits, oral	0, 300, 1000, 3000 mg/kg bw/day	**Maternal NOAEL** 300 mg/kg bw/day Maternal NOEL 300 mg/kg bw/day Developmental NOAEL ≥3000 mg/kg bw/day	**One dam died** **Three abortions occurred** **↓Body weight gain** **↓Food consumption** ↓ Body weight ↓Kidney weight ↓Spleen weight Not teratogenic
Brusick et al., 1981 Dominant lethal assay in rats, oral	0, 65.5, 208.3, 625 mg/kg bw/day (5 days)	NOEL 625 mg/kg bw/day	

A three-generation rat reproduction study conducted with tolclofos-methyl did not reveal evidence of reproduction toxicity.

Reproduction:

Rats: During the 15-week growth periods, parental body weights, growth rates, and total food consumption were comparable between treated and control groups. Mean maternal body weights and body weight changes during gestation and lactation were comparable among groups of each generation. Growth rates of the high-dose P2 males, and the mid- and high-dose P3 males were similar to the respective controls. Increases in mean ovary weights were noted in the high-dose P3 non-pregnant females, but no abnormal histomorphologic alterations were found in the ovaries and no alterations were noted in the remaining data.

Pregnancy rates, fertility rates and parturition indices were generally comparable among groups of each generation.

There were no differences in any of the offspring viability and survival data that were attributable to treatment. Gross pathology findings for the offspring of all generations were considered incidental in nature and showed no relation to compound administration. Increased ovary weights were noted for the F1b pups at all three treatment levels. However, this was not observed for the F2b or F3b pups. Microscopic evaluation did not reveal any compound-related histomorphologic alterations in the tissues examined.

Since the results of the reproduction study provide satisfactory information and show the absence of effects on reproduction, supplementary studies are not considered to be necessary for better interpretation.

There were no dominant lethal effects induced by tolclofos-methyl.

Teratogenicity:

Rat: The mean body weight gain was reduced at 1000 mg/kg bw/day, indicating slight maternal toxicity at 1000 mg/kg bw/day. The number of foetuses with the 5^{th} and/or 6^{th} sternebrae unossified fluctuated among doses and was significantly greater at 1000 mg/kg bw/day than in the control group. However, the unossification of the 5^{th} sternebae was considered to be a developmental variation and in the variation of the 6^{th} sternebrae there was no dose-response. Neither the type nor frequency of malformations indicated an embryo-toxic or teratogenic response. The lower implantation efficiency observed in the 50 mg/kg bw/day group was not reproduced in the second study and is therefore not considered to be related to treatment.

Rabbits: Teratogenicity studies in rats and rabbits indicated no embryo-toxic or teratogenic effects.

The body weight gains were suppressed in the 1000 mg/kg bw/day group or above, food consumption was decreased and there was a slight decrease in some organ weights (kidney, spleen), showing maternal toxic effect. In addition, one dam died and three abortions occurred. Food consumption was markedly decreased in the dams in which the abortions occurred, and this was a probable cause of the abortions. Skeletal variations included eight lumbar vertebrae and 13th rib or asymmetry of sternebrae, but the incidences of these variations were not significantly increased and there were no dose-response. Neither the type nor frequency of malformations indicated an embryo-toxic or teratogenic response. There were no effects on the degree of ossification.

B.6.7 Delayed neurotoxicity

B.6.7.1 Acute delayed neurotoxicity study in hens

Reference	: Okuno, Y. et al., 1982; Takatsuka, M., 1985b	**Exposure**	: 2 times
Title of study and validations	: Acute delayed neurotoxicity study of S-3349 in hens (Okuno et al.); Validation - Acute delayed neurotoxicity study of S-3349 in hens (Takatsuka)	**Dose**	: 8000 mg/kg bw/day
Test substance	: Tolclofos-methyl (Batch No.: 524, Purity: 97.0%, Specification No. 01	**Vehicle**	: Corn oil (32 ml/kg bw)
Administration way	: Oral *via* gavage	**GLP statement**	: No (Quality assurance according to GLP performed later)
Species	: White Leghorn hens	**Guideline**	: In- house method, in accordance with OECD 418
Group size	: 10/dose	**Acceptability**	: Yes
		NOAEL	: > 8000 mg/kg bw/day

Materials and methods

Tolclofos-methyl was administered twice at an interval of 21 days. The animals were kept under observation for 21 days after the second administration. A control group received twice the vehicle, and a positive control group received twice 500 mg/kg of tri-ortho-cresyl phosphate (TOCP). Samples were taken from all animals for blood cholinesterase determination on the day before the first exposure and on 1st, 8th and 21st day after the first administration. All animals were necropsied at the end of the observation period and organs were preserved for histopathological examination.

Findings

General observations: No dead animals were found in the treated group and no sign of delayed-neurotoxicity such as leg-weakness, ataxia and paralysis were observed in the group treated with tolclofos-methyl, although decreased spontaneous motor activity was noticed 0-5 days after dosing including vehicle control. Besides, in the TOCP group, all animals were sacrificed until Day 37 at the moribund condition after they showed leg paralysis. Second administration with TOCP was not performed because the delayed-neurotoxic symptoms were clearly observed.

Mean body weights in the treated group were comparable to the control group. Severe depression of body weight in TOCP group was noticed after occurrence of paralysis (from 16th day).

Average food consumption in the treated group was lower than in the control group (Table B.6.7.1-1).

Table B.6.7.1-1: Delayed neurotoxicity in hen: Food consumption (g/day/hen)

Dose level	0 mg/kg bw (Corn Oil)	500 mg/kg bw (TOCP)	2 x 8000 mg/kg bw (Tolclofos-methyl)
Days after 1st administration			
Day 0-2	167	117	92.3
Day 2-6	204	119**	97.1**
Day 13-16	161	114	106*
Day 16-20	180	79.3**	112*
Day 23-27	174	90.5[a]	107*
Day 34-37	150	0 [b]	94.5**
Day 37-42	152	-	92.3**

a: Number of animals :5
b: Number of animals :1
**: Significantly different from control ($p \leq 0.05$)*
***: Significantly different from control ($p \leq 0.01$)*

Blood cholinesterase : In the treated group, plasma cholinesterase activity at 1st day was depressed to 42% ($p < 0.05$). However the activity recovered at 21st day (Table B.6.7.1-2).

Table B.6.7.1-2: Delayed neurotoxicity in hen: Plasma cholinesterase activity (IU/l)

Dose level	0 mg/kg (Corn Oil)	2 x 8000 mg/kg (Tolclofos-methyl)
Day after 1st administration		
Day -1	901	982
Day 1	1084	628*[a]
Day 8	889	511
Day 21	911	1166

a: Number of animals: 9
**: Significantly different from control ($p \leq 0.05$)*

Histopathology: No structural changes such as axonal degeneration and demyelination suggestive of tissue damages by tolclofos-methyl were found. Slight changes were observed, which incidence and severity were comparable to those of the control group. On the contrary, marked degenerative changes were often noticed in TOCP animals.

Conclusions NOAEL: ≥8000 mg/kg (highest technically possible dosage) - based on the absence of delayed neurotoxic signs and no histopathological changes such as axonal degeneration and demyelination.

B.6.7.2 Summary on delayed neurotoxicity

Study	Dose levels	NOEL/NOAEL	Targets/main effects
Okuno, Y. et al 1982. Acute delayed neurotoxicity, oral, hen	0 or 8000 mg/kg	**NOEL** ≥8000 mg/kg	↓Transient decrease of plasma cholinesterase levels ↓ food consumption

Tolclofos-methyl showed no acute delayed neurotoxicity in a study with leghorn hens. The NOEL was determined to be 8000 mg/kg bw/day (highest technically possible dosage), based on the absence of delayed neurotoxic signs and no histopathological changes such as axonal degeneration or demyelination.

B.6.8 Further toxicological studies (Annex IIA 5.8)

B.6.8.1 Toxicity studies of metabolites

Studies on metabolites of tolclofos-methyl were not performed, since the metabolites were not considered to be relevant to toxicity in mammals.

B.6.8.2 Supplementary studies on the active substance

B.6.8.2.1 Subcutaneous and intraperitoneal acute toxicity study in rats and mice

Reference	: Segawa, T., 1978	**Exposure**	: Single administration
Title of study	: Acute toxicity of S-3349 in rats and mice	**Dose**	: Subcutaneous toxicity study, rats and mice: 1000, 2000, 3000, 4000, 5000 mg/kg bw/day; Intraperitoneal toxicity study, rats: 1000, 1500, 2000, 2500, 3000, 4000, 5000 mg/kg bw/day; mice: 100, 250, 500, 650, 845, 1000, 2000, 3000 mg/kg bw/day
Test substance	: Test material: Tolclofos-methyl, batch No.: 524, purity: 97.0%, specification No. 01	**Vehicle**	: Corn oil (5 ml/kg bw (rats), 25 ml/kg bw (mice)
Administration way	: Subcutaneous (mice), intraperitoneal (rats and mice)	**GLP statement**	: No
Species	: Sprague-Dawley rats and dd mice	**Guideline**	: In-house method
Group size	: 10/sex/dose	**Acceptability**	: Yes
		LD_{50}	: Subcutaneous: 5000 mg/kg bw/day (rats and mice); Intraperitoneal: 4900 mg/kg bw/day (rats), 1070 mg/kg bw/day (mice)

Findings

Subcutaneous (only mice) and intraperitoneal administration of the test material to each species gave rise to toxic symptoms such as decrease of spontaneous motor activity, irregular respiration, dyspnea, piloerection, incontinence of urine and ataxia of hind limb or whole body. No noteworthy sex difference was observed on the toxicity in rats and mice, by any administration route (Tables 6.8.2.1-1 and 6.8.2.1-2). Species difference was found by intraperitoneal route; mice were more sensitive than rats.

In the necropsy, the formation of granulation tissues and/or residues of oily substance were found in subcutaneous injection site of rats and mice, whereas tissues and organs of animals treated intraperitoneally were grossly normal.

Table B.6.8.2.1-1: Acute subcutaneous toxicity of tolclofos-methyl

	Males			Females		
	Dose (mg/kg)	Cumulative Mortality	Time of death	Dose (mg/kg)	Cumulative Mortality	Time of death
Rat	1000	0	-	1000	0	-
	2000	0	-	2000	0	-
	3000	0	-	3000	0	-
	4000	0	-	4000	0	-
	5000	0	-	5000	0	-
Mouse	1000	0	-	1000	0	-
	2000	0	-	2000	0	-
	3000	0	-	3000	0	-
	4000	2/10*	d1	4000	0	-
	5000	0	-	5000	3/10*	d1

d: Day
**: Number of animals which died/number of animals used*

Table B.6.8.2.1-2: Acute intraperitoneal toxicity of tolclofos-methyl

	Males			Females		
	Dose (mg/kg)	Cumulative Mortality	Time of death (No of animals)	Dose (mg/kg)	Cumulative Mortality	Time of death
Rat	1000	0	-	1000	0	-
	1500	0	-	1500	0	-
	2000	0	-	2000	0	-
	2500	1/10*	day7	2500	1/10*	day5
	3000	3/10*	day2: 1; day5: 2	3000	1/10*	day2
	4000	3/10*	day4	4000	3/10*	day4: 1; day5:2
	5000	5/10*	day4: 1; d5: 1; day7: 3	5000	7/10*	day4: 4; day5: 2; day7: 1
Mouse	100	0	-	100	0	-
	250	0	-	250	0	-
	500	0	-	500	0	-
	650	0	-	650	0	-
	845	0	-	845	0	-
	1000	2/10	day1: 2; day2: 1; day3: 2	1000	1/10	day1: 2; day2: 1; day4: 1
	2000	3/10	day1	2000	2/10	day1
	3000	6/10	day1: 9; day2: 1	3000	6/10	day1: 4; day2: 6

**: Number of animals which died/number of animals used*

Conclusions By the subcutaneous route, tolclofos-methyl showed toxicity from 2000 mg/kg bw and above in mice; the LD_{50} was greater than 5000 mg/kg bw.
By the intraperitoneal route, tolclofos-methyl showed toxicity at 650 mg/kg bw in mice and from 2000 mg/kg in rats; the LD_{50}s were about 5000 and 4900 mg/kg bw for male and female rats respectively, and 1070 and 1260 mg/kg bw for male and female mice, respectively.

B.6.9 Medical data and information (Annex IIA 5.9)

B.6.9.1 Medicinal surveillance on manufacturing plant personnel: Medical examination of factory workers

Reference	: Murayama, F., 1991
Title of study	: A review of medical examination of factory workers exposed to tolclofos-methyl
Test substance	: Tolclofos-methyl, Batch No.: Not specified, Purity: Not specified, Specification No. 01

Materials and methods

All the workers (20 males) who have been engaged in the packaging of technical tolclofos-methyl into drums have been considered in this survey. The following parameters were checked: average daily time spent on packaging, career of the packing operation, worker age, personal protection used by the workers. Re-examination of clinical records of the workers were performed on the last three years (records for 5 occasions, at about 6-month intervals).

Findings

The manufacturing schedule means that each worker spends 4 hours on packaging. All workers have consecutively engaged in the operations since manufacturing began in 1988. Workers age as of August 1991 was as follows:

Table 6.9.1-1: Workers' age (as of August, 1991)

Age (years)	No. of workers
30 - 39	2
40 - 49	12
50 - 59	6

Workers wear one-piece worksuit, plastic helmets, glasses, gloves, dust mask with charcoal filter and workboots with protective toe caps.

In the re-examination of the records for the 20 workers, no abnormalities were observed.

Conclusions Re-examination of clinical records confirmed that no occupation related problems were observed or reported for technical tolclofos-methyl.

B.6.10 Summary of mammalian toxicology and proposed ADI, AOEL, ARfD and drinking water limit (AnnexIIA 5.10)

B.6.10.1 Absorption, distribution, excretion and metabolism (toxicokinetics)

Absorption

Most of the ^{14}C tolclofos-methyl-derived radioactivity was rapidly eliminated by male and female rats at both high (200 mg/kg) and low (5 mg/kg) dose levels, and by male and female mice at the low dose level. Most of the radioactivity was eliminated within the first 48 hours. The similarities in elimination rates for all groups and the fact that urinary excretion is the primary route of elimination, indicate that the processes of absorption and elimination are relatively unaffected at dosages up to 200 mg/kg bw. The absorption from the gastrointestinal tract relatively rapid and is estimated to be higher than 78% based on the biliary excretion study, which indicated that the unabsorbed tolclofos-methyl was excreted without any metabolic transformation in the gastrointestinal tract.

Distribution

Distribution of tolclofos-methyl into tissues is small and persistence of the compound over a long period is unlikely.

Excretion

Tolclofos-methyl was readily excreted in rats and mice, mainly in the urine. Less than 1% of the dose was retained in the tissues after 7 days. Excretion into bile in the bile-duct cannulated rats showed that cumulative excretion over 48 hours was 5.8 to 11.7% of the dose of ^{14}C in the bile, 46.7 to 59.4% in the urine and 42.3 to 23.7% in the feces, in males and females respectively (dose level: 5 mg/kg). Only the parent compound was detected in feces and bile when collected 0 to 24 hours after administration. The unabsorbed tolclofos-methyl was therefore supposed to be excreted without being degraded in the gastrointestinal tract.

Metabolism

In both rats and mice, tolclofos-methyl was mainly metabolised via oxidative desulfuration of the P=S group to P=O, oxidation of 4-methyl group, and cleavage of P-O-aryl and P-O-methyl linkages.

The major metabolite in both rats and mice was 3,5-dichloro-4-hydroxybenzoic acid. This metabolite was excreted as the glycine conjugate in mice and as the free form in rats.

The proposed metabolic pathway for tolclofos-methyl in rats and mice is shown in Figure B.6.1.2-1