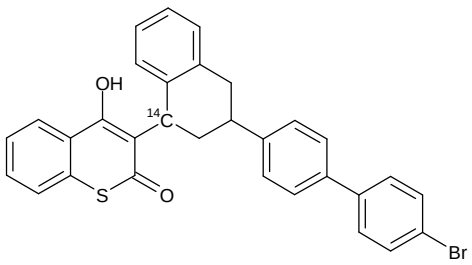
Section A 7.1.1.1.1 Annex Point IIA VII.7.6.2.1		Hydrolysis as a function of pH and identification of breakdown products	
		1 REFERENCE	**Official use only**
1.1	**Reference**	Xxxxx, X., XXXX, Determination of the hydrolysis rate constants of XXXXXX. XXXXXXXXXXXXX, laboratory report no. xxxx, xx Xxxxxxxxx XXXX (unpublished). Section no.: A 7.1.1.1.1-01.	
1.2	**Data protection**	Yes.	
1.2.1	Data owner	LiphaTech S.A.S.	
1.2.2	Companies with letter of access	None.	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes. The study was performed to US EPA Pesticide Assessment Guidelines, Subdivision N, Paragraph 161-1.	
2.2	**GLP**	Yes.	
2.3	**Deviations**	Yes. The study was not conducted to the tiered approach outlined by the recommended guideline (EC method C.7, OECD 111); however, the study provides sufficient information on the hydrolytic behaviour of the test substance.	
		3 MATERIALS AND METHODS	
3.1	**Test material (radiolabelled)**	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one. Referred to as XXXXXX in the study report.	
3.1.1	Lot/Batch number	Code no. XXXXXXXX, lot no. XXX.	
3.1.2	Specification	Specific activity 23 mCi/mmol.	
3.1.3	Purity	RCP (radiochemical purity) > 96% by two TLC systems.	
3.1.4	Further relevant properties	Position of radiolabel given below: 	

Section A 7.1.1.1.1 **Annex Point IIA VII.7.6.2.1**		**Hydrolysis as a function of pH and identification of breakdown products**	
3.2	**Test material (non-radiolabelled)**	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one. Referred to as XXXXXX in the study report.	
3.2.1	Lot/Batch number	Not specified.	
3.2.2	Specification	No further details.	
3.2.3	Purity	Not specified.	
3.2.4	Further relevant properties	Not applicable.	
3.3	**Testing procedure**	The rate of hydrolysis of difethialone in sterile aqueous buffer at a concentration of approximately 1 mg/L and at a temperature of 25°C was investigated at pH values 5, 7 and 9.	
3.3.1	Test system	Acidic, neutral and alkaline buffer solutions were prepared using distilled, deionised water as described in Table A 7.1.1.1.1-1. All buffer solutions were sterilised by filtration (0.22 µm). The treatment and incubation of the test solutions is summarised in Table A 7.1.1.1.1-2. Difethialone was prepared in the test samples at a concentration of 1 mg/L. Further details of the test system is provided in Table A 7.1.1.1.1-3.	**x**
3.3.2	Temperature	25°C.	
3.3.3	pH	The study was conducted at pH values of 5, 7 and 9. Measurements were taken at each sampling interval, see Table A 7.1.1.1.1-4, to confirm that the pH of the buffer solutions was maintained throughout the study.	
3.3.4	Duration of the test	30 days.	
3.3.5	Number of replicates	Duplicate samples for each pH at each sampling interval.	
3.3.6	Sampling	Samples were taken for analysis at 0, 1, 7, 14, 22 and 30 days. At each sampling interval methanol (10 mL) was added to the buffer solutions to recover radioactivity adsorbed to glass surfaces. The level of radioactivity in the buffer solutions was quantified (50µL) by LSC and an aliquot taken for TLC analysis.	
3.3.7	Analytical methods	For routine chromatographic analysis the buffer solutions were analysed by two different TLC systems using silica plates (0.25 mm). TLC plates were either developed in hexane/ ether/ acetic acid (25:70:5 v/v/v) or methanol/ acetic acid (90/10 v/v). Non radiolabelled reference standards were visualised using UV light (254 nm). Radioactive regions were quantified using a linear analyser. The RCP determinations were similarly conducted using hexane/ ether/ acetic acid (25/70/5 v/v/v) and methanol/ acetic acid (90/10 v/v).	
		4 RESULTS	

Section A 7.1.1.1.1 Annex Point IIA VII.7.6.2.1		Hydrolysis as a function of pH and identification of breakdown products	
4.1	**Recovery of applied radioactivity**	The recovery of applied radioactivity from the buffer solutions is summarised in Table A 7.1.1.1.1-4. The amount of applied radioactivity recovered ranged from 83 to 100% (average 96%). Consequently, any evolved volatile components were not significant. At one sampling interval (22 days) the recovery of applied radioactivity declined to approximately 83%, however, by the end of the study (30 days) the level had recovered to 93%. Generally the recovery of applied radioactivity was 90% or greater and is considered acceptable for the purposes of this study and is not thought to have effected the results and conclusions. The pH of the test solutions was maintained throughout the study.	
4.2	**Profile of components**	The level of difethialone observed in the sterile aqueous buffer solutions is summarised in Table A 7.1.1.1.1-5. At a temperature of 25°C very little hydrolysis was observed. Examples of chromatographic analysis given in the study report do not show any significant degradation products. At the molarities specified, any buffer effects were not thought to have significantly affected the hydrolysis rates observed.	**x**
4.3	**Hydrolysis rate constant (k_h)**	The hydrolysis rate of difethialone was recalculated using the procedure described in Section A 7.2.1-01. The recalculation of the DT_{50} and DT_{90} values is presented graphically in Figures A 7.1.1.1.1-1 to 7.1.1.1.1-3 and summarised in Table A 7.1.1.1.1-5. The hydrolysis of difethialone at a temperature of 25°C in sterile aqueous buffer solution at pH values of 5, 7 and 9 did not give a good correlation to first-order kinetics (R^2 values were in the range 0.11 to 0.35). The best fit DT_{50} values of difethialone were determined to be 516 days (pH 5), 175 days (pH 7) and 155 days (pH 9). The corresponding DT_{90} values were determined to be 1714 days (pH 5), 580 days (pH 7) and 515 days (pH 9).	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	The hydrolytic behaviour of difethialone was investigated in sterile aqueous buffer (pH value 5, 7 and 9) at a temperature of 25°C. The GLP study was conducted to US EPA Guidelines 161-1 in 1986.	
5.2	**Results and discussion**	The recovery of the applied radioactivity ranged from 83 to 100%. The pH of the buffer solutions was maintained throughout the duration of the study. No significant hydrolysis products were observed.	
5.2.1	k_H	The hydrolysis rate constants were calculated to be 0.001343 day^{-1} (pH 5), 0.003967 day^{-1} (pH 7) and 0.004469 day^{-1} (pH 9), see Table A 7.1.1.1.1-5.	
5.2.2	DT_{50}	The DT_{50} values were 516 days (pH 5), 175 days (pH 7) and 155 days (pH 9), see Table A 7.1.1.1.1-5.	
5.2.3	r^2	The observed hydrolysis did not give a good correlation to first-order kinetics (R^2 = 0.11 to 0.35).	

Section A 7.1.1.1.1 **Annex Point IIA VII.7.6.2.1**	**Hydrolysis as a function of pH and identification of breakdown products**	
5.3 Conclusion	Although not conducted to the tiered approach, as recommended in the EU guideline, the data generated is sufficient to estimate the hydrolytic properties of difethialone. Difethialone is stable to hydrolysis under acidic conditions (pH 4) with an estimated half-life of > 1 year. Under neutral (pH 7) and alkaline (pH 9) conditions difethialone is slowly hydrolysed with estimated DT50 values of 175 and 155 days, respectively. No significant degradation products were formed. The hydrolytic degradation of difethialone is not considered to be a significant process in the environment.	**x**
5.3.1 Reliability	2.	
5.3.2 Deficiencies	Yes The study contained some deficiencies when compared to modern day standards. These deficiencies are discussed in more detail where appropriate under the relevant headings above and are not considered to have adversely affected the quality of the results.	

Section A 7.1.1.1.1 Annex Point IIA VII.7.6.2.1	Hydrolysis as a function of pH and identification of breakdown products	
Evaluation by Competent Authorities		
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	14 December 2004; revised 31 October 2006	
Materials and Methods	Agree with applicant's version **Comments (3.3.1):** Test concentrations were slightly above the water solubility of 0.39 mg/l.	
Results and discussion	Agree with applicant's summary and conclusion **Comments (4.2):** The extent of hydrolysis observed after 30 days at a temperature of 25°C was 4.9%, 11.2% and 10.4%, respectively (table A 7.1.1.1.1-5). The identification of breakdown products accounting for more than 10 % of active substance added should have been performed. However, examples of chromatographic analysis given in study A7.1.1.1.1 do not show any significant degradation products. It is therefore anticipated that the 10.4 %and 11.2 % degradation observed at pH 9 and 7, respectively, comprises of several minor metabolites < 10% and do not represent single metabolites, because these would have been clearly visible in the chromatograms. No information is available on these minor metabolites. However, hydrolysis of difethialone is slow and due to the low water solubility of difethialone and its strong adsorption tendency to organic matter hydrolysis is not considered to play a major role in the environment.	
Conclusion	**Comment (5.3):** Although not conducted according to the tiered approach, as recommended in the EU guideline, the data generated is sufficient to estimate the hydrolytic properties of difethialone. Difethialone is stable to hydrolysis under acidic conditions (pH 5) with an estimated half-life of > 1 year. Under neutral (pH 7) and alkaline (pH 9) conditions difethialone is slowly hydrolysed with estimated DT50 values of 175 and 155 days, respectively. Degradation products were formed but not identified and the very slow hydrolysis rate indicates that it is the parent compound that at all times will be the main compound of concern.	
Reliability	2	
Acceptability	Acceptable with the deficiencies noted above	
Remarks		

Table A 7.1.1.1.1-1: Type and composition of buffer solutions

pH	Type of buffer (final molarity)	Composition
5	Acetate buffer (0.01M)	0.01M Sodium acetate adjusted to pH 5.0 (+/- 0.05) with 0.1M acetic acid.
7	Phosphate buffer (0.007M)	0.067M Sodium dihydrogen phosphate, NaH_2PO_4 (30 mL) mixed with 0.067M dipotassium hydrogen phosphate, K_2HPO_4 (61 mL) and diluted tenfold.
9	Borate buffer (0.025M)	0.025M Disodium tetraborate, $Na_2B_4O_7$ adjusted to pH 9.0 (+/- 0.05) with 0.1M acetic acid.

Table A 7.1.1.1.1-2: Description of test solution

Criteria	Details
Purity of water	Distilled, deionised.
Preparation of test medium	Test substance (ca 0.25 mg), dissolved in chloroform (13 µL), was reconstituted with DMF (1.5 mL) and then diluted with buffer solution (148.5 mL) in an Erlenmeyer flask (250 mL).
Test concentrations (mg a.i./L)	1 ppm.
Temperature (°C)	25 ± 1°C.
Controls	Not applicable.
Identity and concentration of co-solvent	Dimethylformamide (DMF) 1% v/v.
Replicates	Two replicates for each pH value at each sampling interval.

Table A 7.1.1.1.1-3: Description of test system

Glassware	The bulk treated buffer solution was prepared in Erlenmeyer flasks (250 mL). The individual sub-samples (10 mL) were incubated in screw capped glass vials (20 mL) covered in foil to exclude light.
Other equipment	Incubator (Ambi-Hi-Low Lab-Line). pH measurements (Beckman Expandomatic pH meter). Filtration (Gelman filtration apparatus, metricel membrane). Liquid scintillation counter (Packard Tri-Carb 3380). TLC linear analyser (Berthold Instruments Inc. LB 2832 TLC linear analyser and LB 500 data acquisition system).
Method of sterilization	The buffer solutions were sterilised by filtration (0.22 µm). The glassware was sterilised by autoclaving for 60 minutes at 15 psig and 121°C.

Table A 7.1.1.1.1-4: Recovery of applied radioactivity from sterile aqueous buffer

Incubation time (days)	**Recovery of applied radioactivity (mg equiv/L)**					
	pH 5[1]		**pH 7[2]**		**pH 9[3]**	
	Measured	**Mean**	**Measured**	**Mean**	**Measured**	**Mean**
0	0.92	0.92 (100%)	0.99	0.99 (100%)	1.07	1.07 (100%)
1	0.90, 0.90	0.90 (98%)	0.98, 0.97	0.98 (99%)	1.04, 1.05	1.05 (97%)
7	0.86, 0.89	0.88 (96%)	0.95, 1.00	0.98 (99%)	0.98, 1.05	1.02 (95%)
14	0.90, 0.93	0.92 (100%)	0.98, 1.00	0.99 (100%)	1.01, 1.08	1.05 (97%)
22	0.76, 0.76	0.76 (83%)	0.92, 0.95	0.94 (95%)	0.95, 1.01	0.98 (92%)
30	0.84, 0.87	0.86 (93%)	0.82, 0.92	0.87 (88%)	0.97, 0.97	0.97 (91%)

Values in parenthesis are percentages of initial concentrations (nominal concentration 1 mg/L).

The overall range of total radioactivity recovered from the samples was 83 to 100% (mean 96%).

[1] Measured pH values 5.05 at 0 days, 5.10 at 30 days. Range 5.00 to 5.20 (mean 5.08).
[2] Measured pH values 6.95 at 0 days, 7.40 at 30 days. Range 6.95 to 7.60 (mean 7.34).
[3] Measured pH values 8.95 at 0 days, 9.00 at 30 days. Range 8.95 to 9.00 (mean 9.00).

Table A 7.1.1.1.1-5: Concentration of difethialone in sterile aqueous buffer

Incubation time (days)	Concentration of difethialone in buffer solution (mg/L)					
	pH 5		pH 7		pH 9	
	Measured	Mean	Measured	Mean	Measured	Mean
0	0.82, 0.81	0.82	0.84, 0.94	0.89	0.94, 0.98	0.96
1	0.80, 0.83	0.82	0.82, 0.89	0.86	0.89, 1.01	0.95
7	0.73, 0.86	0.80	0.86, 0.95	0.90	0.85, 0.97	0.91
14	0.86, 0.78	0.82	0.88, 0.88	0.88	0.96, 0.90	0.93
22	0.62, 0.59	0.60	0.86, 0.72	0.79	0.86, 0.75	0.81
30	0.77, 0.78	0.78 (4.9%) [1]	0.78, 0.80	0.79 (11.2%) [1]	0.88, 0.85	0.86 (10.4%) [1]

[1] Values in parenthesis relate to the percentage of degradation observed after 30 days at a temperature of 25°C.

Figure A 7.1.1.1.1-1: Re-calculation of DT_{50} and DT_{90} values for hydrolysis of difethialone in sterile aqueous buffer (pH 5)

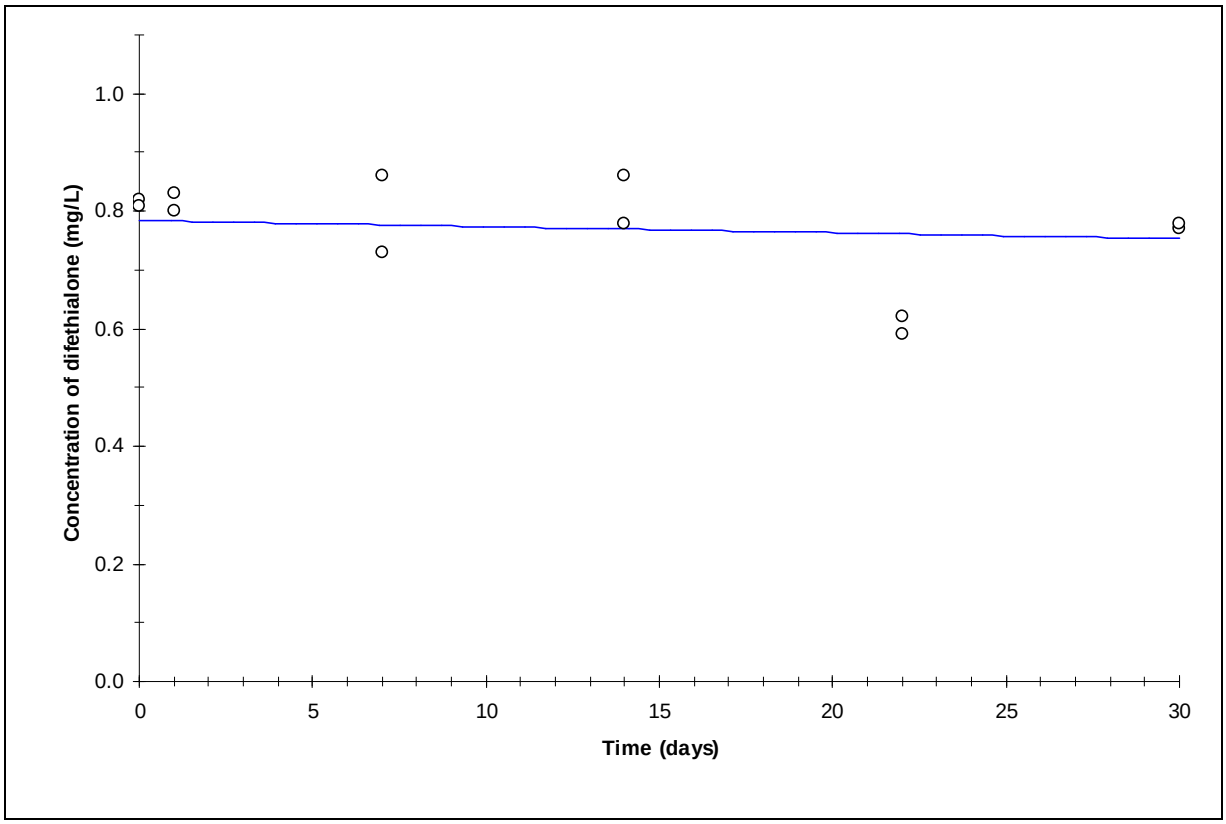


Figure A 7.1.1.1.1-2: Re-calculation of DT_{50} and DT_{90} values for hydrolysis of difethialone in sterile aqueous buffer (pH 7)

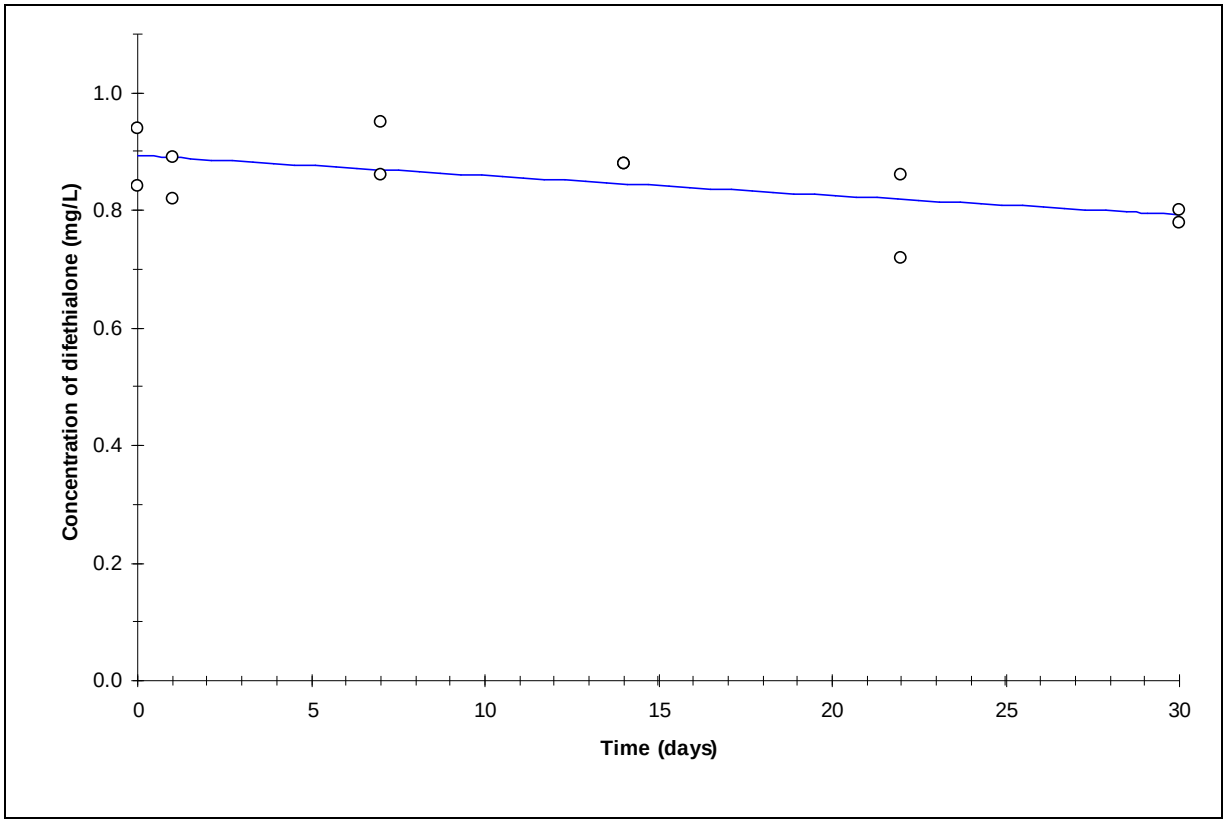


Figure A 7.1.1.1.1-3: Re-calculation of DT_{50} and DT_{90} values for hydrolysis of difethialone in sterile aqueous buffer (pH 9)

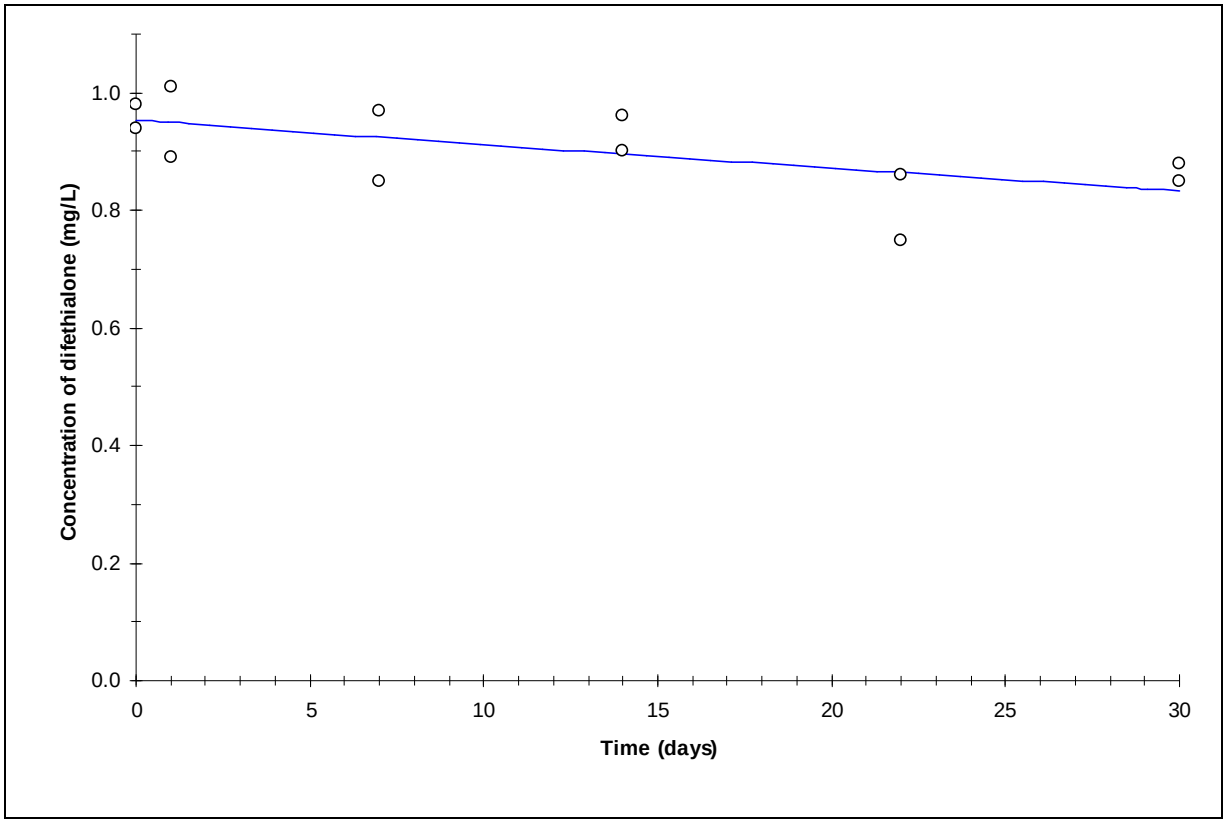
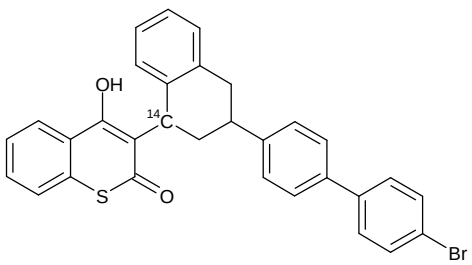


Table A 7.1.1.1.1-6: First order DT_{50} and DT_{90} values for the rate of hydrolysis of difethialone in sterile aqueous buffer

Buffer	Data range (days)	$DT_{50(lab)}$ (days)	$DT_{90(lab)}$ (days)	Regression parameters		
				C_0	k	R^2
pH 5	0 to 30 [1]	516 [2]	1714 [2]	0.7841	0.001343	0.11
pH 7	0 to 30 [1]	175 [2]	580 [2]	0.8935	0.003967	0.35
pH 9	0 to 30 [1]	155 [2]	515 [2]	0.9534	0.004469	0.39

[1] All data points were used.

[2] DT_{50} (or DT_{90}) value was not demonstrated experimentally, result obtained by extrapolation.

Section A 7.1.1.1.2 **Annex Point IIA VII.7.6.2.2**	**Phototransformation in water including identity of transformation products**	
	1 REFERENCE	**Official use only**
1.1 Reference	Xxxxx, X., XXXXx, Determination of the solution photolysis rate of LM2219 (Difethialone). XXXXXX. XXXXXXXXXXXXX, laboratory report no. xxxx, xx Xxxxxxxxx XXXX (unpublished). Section no.: A 7.1.1.1.2-01.	
1.2 Data protection	Yes.	
1.2.1 Data owner	LiphaTech S.A.S.	
1.2.2 Companies with letter of access	None	
1.2.3 Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
	2 GUIDELINES AND QUALITY ASSURANCE	
2.1 Guideline study	Yes. The study was performed to US EPA Pesticide Assessment Guidelines, Subdivision N, Paragraph 161-2.	
2.2 GLP	Yes.	
2.3 Deviations	No. Apart from minor deviations, the study meets the requirements of the recommended guideline (SETAC, OPPTS 835.2210).	
	3 MATERIALS AND METHODS	
3.1 Test material	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one. Referred to as LM2219 in the study report.	
3.1.1 Lot/Batch number	Code no. XXXXXXX, lot no. XXX	
3.1.2 Specification	Specific activity 23 mCi/mmol.	
3.1.3 Purity	RCP (radiochemical purity) > 96% by two TLC systems.	
3.1.4 Radiolabelling	Position of radiolabel given below: 	

Section A 7.1.1.1.2 Annex Point IIA VII.7.6.2.2		Phototransformation in water including identity of transformation products	
3.1.5	Further relevant properties	None	
3.2	**Test material (non-radiolabelled)**	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one. Referred to as LM2219 in the study report.	
3.2.1	Lot/Batch number	Not specified.	**x**
3.2.2	Specification	No further details.	**x**
3.2.3	Purity	Not specified.	**x**
3.2.4	Further relevant properties	Not applicable.	
3.3	**Reference substances**	No reference substances were used.	
3.4	**Testing procedure**	The rate of photolysis of difethialone in aqueous solution was investigated under natural sunlight at pH values of 5, 7 and 9.	**x**
3.4.1	Test system	Acidic, neutral and alkaline buffer solutions were prepared using distilled, deionised water as described in Table A 7.1.1.1.2-1. All buffer solutions were sterilised by filtration (0.22 µm). All glassware were sterilised by autoclaving for 60 minutes at 15 psig and 121°C. The treatment and incubation of the test solutions is summarised in Table A 7.1.1.1.2-2. Further details of the test system and equipment used is provided in Table A 7.1.1.1.2-3.	
3.4.2	Properties of light source	Natural sunlight, see Table A 7.1.1.1.2-3.	
3.4.3	Determination of irradiance	Sunlight measurements were made using a Blak-Ray Long wave ultraviolet meter (Ultraviolet Products Inc., San Gabriel, CA, USA).	
3.4.4	Temperature	Ambient temperature under natural sunlight, temperature measurements were in the range 28 to 35°C for the duration of the test.	
3.4.5	pH	The study was conducted at pH values of 5, 7 and 9. Measurements were taken at the beginning and end of the exposure period (1.5 hours duration), see Table A 7.1.1.1.2-4, to confirm that the pH of the buffer solutions was maintained throughout the study.	
3.4.6	Duration of the test	The definitive phase of the study was conducted over 1.5 hours.	
3.4.7	Number of replicates	Duplicate exposed samples for each pH at each sampling interval. Single dark control samples were taken for each pH at each sampling interval.	
3.4.8	Sampling	Samples were taken for analysis at intervals of 0, 10, 20, 30, 45, 60 and 90 minutes. At each sampling interval the level of radioactivity in the buffer solutions was quantified by LSC and an aliquot (25 µL) taken for	

Section A 7.1.1.1.2 **Annex Point IIA VII.7.6.2.2**		**Phototransformation in water including identity of transformation products**	
		TLC analysis. Sunlight measurements, weather observations and ambient temperatures were recorded at each sampling interval.	
3.4.9	Analytical methods	For routine chromatographic analysis the buffer solutions were analysed by two different TLC systems using silica plates (0.25 mm). TLC plates were either developed in hexane/ ether/ acetic acid (25:70:5 v/v/v) or methanol/ acetic acid (90/10 v/v). Non radiolabelled reference standards were visualised using UV light (254 nm). Radioactive regions were quantified using a linear analyser. The RCP determinations were similarly conducted using hexane/ ether/ acetic acid (25/70/5 v/v/v) and methanol/ acetic acid (90/10 v/v).	
3.5	**Transformation products**		
3.5.1	Method of analysis for transformation products	The levels of difethialone and corresponding degradation products were monitored using the chromatographic systems described in Section 3.4.9.	
		4 RESULTS	
4.1	**Screening test**	Some preliminary tests were performed using the same methodologies as described above, however these were used solely as range finders (i.e. to determine the required exposure duration etc.) and the results are not described in detail in the study report.	
4.2	**Actinometer data**	A chemical actinometer was not used for this study; sunlight intensity measurements were made using an ultraviolet meter.	
4.3	**Photolysis data**		
4.3.1	Recovery of applied radioactivity, mass balance	The recovery of applied radioactivity from the exposed samples and dark controls is summarised in Table A 7.1.1.12.-4. The amount of applied radioactivity recovered from the exposed samples and dark controls ranged from 82.8 to 106.2% (overall average 98.0, 92.4 and 96.4% at pH values of 5, 7 and 9 respectively) and 78.8 to 102.7% (overall average 100.0, 86.9 and 87.6% at pH values of 5, 7 and 9 respectively), indicating a complete mass balance. No attempt was made to recover evolved volatile components. As a complete mass balance was achieved, the amount of any evolved volatile components could not have been significant (i.e. > 10% AR). Measurements of the pH of the buffer solutions at the beginning and end of the incubation period indicated that the pH of the solutions was maintained over the duration of the test period.	**x**

Section A 7.1.1.1.2 **Annex Point IIA VII.7.6.2.2**		**Phototransformation in water including identity of transformation products**	
4.3.2	Concentration values	The concentration of difethialone in the buffer solutions (expressed as a percentage of the initial concentration) exposed to natural sunlight and for the dark controls, at each sampling interval, is summarised in Table A 7.1.1.1.2-5 and Table A 7.1.1.1.2-6, respectively. Two chromatography systems (SS 1 and SS 2) were used to analyse the buffer solutions. Values obtained using SS 2 are consistently higher than that obtained using SS 1, indicating incomplete resolution of difethialone from degradation products using SS 2.	
4.3.3	Photolysis rate constant, k^c_p	The photolysis of difethialone under natural sunlight was rapid at all pH values. After 90 minutes the amount of difethialone comprised 30.8 to 39.3% of applied radioactivity. The recalculation of the DT_{50} and DT_{90} values is presented graphically in Figures 7.1.1.1.2-1 to 7.1.1.1.2-3 and summarised in Table A 7.1.1.1.2-7. The best fit DT_{50} values for the photolysis of difethialone in buffer solutions at pH 5, 7 and 9 were determined to be 59.7, 61.9 and 54.5 mins respectively (overall average 58.7 mins).	**x**
4.3.4	Kinetic order	The photolysis of difethialone, under natural sunlight, gave a good correlation to pseudo first order kinetics (R^2 values were in the range 0.78 to 0.91).	
4.3.5	Reaction quantum yield (ϕ^c_E)	The sunlight reaction quantum yield (ϕ^c_E) of the test substance was not determined.	**x**
4.4	**Specification of the transformation products**	Photolysis of difethialone in aqueous sterile buffer led to the formation of two significant components. The transformation products were not fully identified and are referred to as "origin material" and "Rf 0.8". The maximum levels of Origin and Rf 0.8 observed were 39.0 and 23.2% AR, respectively. A summary of the levels observed is presented in Table A 7.1.1.1.2-8. Investigation into the degradation products was not comprehensive, however, it can be said that photolysis of difethialone led to the formation of two areas of radioactivity. The chromatographic analysis of these areas of radioactivity was not sufficient to resolve whether the areas were due to individual degradation products or multiple components or sufficient to identify the components structurally.	**x**
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	The rate of photolysis of difethialone in aqueous solution was investigated under natural sunlight at pH values of 5, 7 and 9. The GLP study was conducted to US EPA Guideline 161-2 in 1986.	
5.2	**Results and discussion**	The recovery of applied radioactivity ranged from 82.8 to 106.2% (overall average 96%). No attempt was made to collect evolved volatile components, however as a mass balance was obtained the level of volatile components, if any, was not significant. The pH of the buffer solutions was maintained throughout the incubation period. Photolysis of difethialone under natural sunlight was rapid in buffer solutions at each pH value. Photolysis gave a good correlation to pseudo first order kinetics.	

Section A 7.1.1.1.2 **Annex Point IIA VII.7.6.2.2**	**Phototransformation in water including identity of transformation products**	
5.2.1 k^c_p	The corresponding rate constants ranged from 0.01271 to 0.01120 min^{-1}.	
5.2.2 ϕ^c_E	The quantum yield was not determined.	
5.2.3 $t_{1/2E}$	The DT_{50} values ranged from 54.5 to 61.9 mins (overall average 58.7 mins).	
5.3 Conclusion	Photolysis of difethialone in aqueous solution is rapid. No pH effects were observed.	**x**
5.3.1 Reliability	2	
5.3.2 Deficiencies	Yes The study contained some deficiencies when compared to modern day standards. These deficiencies are discussed in more detail where appropriate under the relevant headings above and are not considered to have adversely affected the quality of the results. In addition, the study made no attempt to identify the photolysis components formed in significant quantities (i.e. > 10% AR). As the study is conducted for classification purposes only (i.e. actual use of the biocidal products will not result in exposure to aquatic systems) the identity of the photolysis components is not considered relevant.	
Evaluation by Competent Authorities		
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	22 December 2004; revised 31 October 2006	
Materials and Methods	Agree with applicant's summary and conclusion. **Comments (3.2.1, 3.2.2 and 3.2.3):** Should have been filled with relevant information available in the study report. **Comments (3.4)** The test concentration should have been included and a remark made that the tested concentration of 1 mg/l is above the solubility of difethialone.	

Section A 7.1.1.1.2 Annex Point IIA VII.7.6.2.2	Phototransformation in water including identity of transformation products	
Results and discussion	Agree with applicant's summary and conclusion. **Comments (4.3.1)** Recovery was quite variable as indicated in Table A 7.1.1.1.2-4 and may be due to the mentioned test concentration which was above the determined solubility limit. A reduction in amount recovered was most noticeable in dark controls at pH7 and pH 9. **Comments (4.3.3)** Although a significant loss of test material was observed in the dark controls no attempt was made to correct the estimated photolysis rate for losses in dark controls. **Comments (4.3.5)** The study did not include a quantum yield and this makes application of the DT50 values for risk assessment less applicable. As alternative one may take into account that the test was performed in September in USA ($39^{o}25$'N). These deficiencies are accounted for in A 7.1.1.1.2-02 which includes estimation of quantum yield. **Comments (4.4)** Transformation products accounting for 39. 0 and 23.3 % AR respectively were observed but not identified as required according to Annex Point IIA VII.7.6.2.2 According to the TNsG any metabolites or other degradation products that at any sampling time during the studies account for more than 10% of the active substance added should be identified and their degradation rates should be studied. However, as the water compartment is not the main compartment of risk no further testing is required. Moreover, the high adsorption tendency of difethialone to organic matter further reduces difethialone concentration in water bodies. Photodegradation in the environment only takes place in the upper layers of surface water. Laboratory results, carried out with distilled or tap water, do not represent environmental conditions, where surface water is normally deeper and muddier. Considering the total water body photodegradation is therefore not considered to be a relevant process for difethialone dissipation in the environment and this justifies also why further testing is not considered necessary.	
Conclusion	**Comment (5.3):** Photolysis of difethialone in aqueous solution is rapid. Transformation products accounting for 39. 0 and 23.2 % AR respectively were observed but not identified. As the water compartment is not the main compartment of risk no further testing is required. No pH effects were observed.	
Reliability	2	
Acceptability	Acceptable with the restrictions noted above.	
Remarks	Photolysis is rapid based on this study but the determined DT50 is considered very uncertain. A new study is not required as a newer study (A 7.1.1.1.2-02) is found acceptable.	

Table A 7.1.1.1.2-1: Type and composition of buffer solutions

pH	Type of buffer (final molarity)	Composition
5	Acetate buffer (0.01M)	0.01M Sodium acetate adjusted to pH 5.0 (+/- 0.05) with 0.1M acetic acid.
7	Phosphate buffer (0.007M)	0.067M Sodium dihydrogen phosphate, NaH_2PO_4 (30 mL) mixed with 0.067M dipotassium hydrogen phosphate, K_2HPO_4 (61 mL) and diluted tenfold.
9	Borate buffer (0.025M)	0.025M Disodium tetraborate, $Na_2B_4O_7$ adjusted to pH 9.0 (+/- 0.05) with 0.1M acetic acid.

Table A 7.1.1.1.2-2: Description of test solution and controls

Criteria	Details
Purity of water	Distilled, deionised.
Preparation of test medium	Test substance (*ca* 0.025 mg), dissolved in chloroform (2.25 µL), was reconstituted with dimethylformamide, DMF (250 µL) and then diluted with buffer solution (25 mL) in a glass scintillation vial (25 mL). Aliquots (7 mL) of the treated solutions were added to stoppered (Teflon lined) quartz glass tubes. For each pH value at each sampling interval, two tubes were prepared for exposure to sunlight and a further tube was prepared and wrapped in tin foil for use as a dark control. From the times specified for the treatment in the report, it appears that the study was conducted over 2 days, i.e. not all the samples were treated at the same time.
Test concentrations (mg a.i./L)	1 ppm (mg/L).
Temperature (°C)	Ambient temperature, temperature measurements were in the range 28 to 35°C.
Controls	Dark control samples were similarly prepared.
Identity and concentration of co-solvent	Dimethylformamide (DMF) 1% v/v.
Replicates	Duplicate exposed samples for each pH at each sampling interval. Single dark control samples were taken for each pH at each sampling interval.

Table A 7.1.1.1.2-3: Description of test system

Criteria	Details
Glassware	The bulk treated buffer solution was prepared in Erlenmeyer flasks (250 mL). The treated samples were prepared in glass scintillation vials (25 mL) and aliquots (7 mL) were transferred to quartz glass tubes.
Other equipment	Filtration (Gelman filtration apparatus, metricel membrane). pH measurements (Beckman Expandomatic pH meter). TLC plates (EM Laboratories Inc.). Liquid scintillation counter (Beckman LS3801). TLC linear analyser (Berthold Instruments Inc. LB 2832 TLC linear analyser and LB 500 data acquisition system).
Method of sterilisation	The buffer solutions were sterilised by filtration (0.22 µm). The glassware was sterilised by autoclaving for 60 minutes at 15 psig and 121°C.
Test apparatus	Exposed samples were maintained exposed to direct natural sunlight in quartz glass tubes, held at an angle 45° to the sun on an open wire support with a dark background approximately 1 foot (30 cm) behind. Dark control samples were wrapped to tin foil to protect from sunlight. The tubes were filled to near capacity to minimise any potential losses due to volatilisation. Light measurements were made using a Blak-Ray Long wave ultraviolet meter (Ultraviolet Products Inc., San Gabriel, CA, USA).
Properties of natural sunlight:	Samples were exposed to natural sunlight.
Latitude	Latitude 39°25' N.
Hours of daylight	Afternoon of the days used.
Time of year	15 and 16 September 1986.
Light intensity	The majority of the exposure period was performed with the sunlight intensity at 5200 to > 6000 µW/cm^2. The lowest recorded intensity was 2600 µW/cm^2.
Solar irradiance (L_λ)	Not measured.

Table A 7.1.1.1.2-4: Recovery of applied radioactivity from sterile aqueous buffer

Incubation time (mins)	Recovery of applied radioactivity (% AR)					
	pH 5[1]		pH 7[2]		pH 9[3]	
	Measured	Mean	Measured	Mean	Measured	Mean
Exposed samples						
0	100.0	100.0	100.0	100.0	100.0	100.0
10	99.1, 106.2	102.7	98.0, 98.0	98.0	99.0, 96.0	97.5
20	99.1, 101.8	100.5	100.0, 90.9	95.5	103.0, 94.9	99.0
30	97.3, 100.9	99.1	94.9, 86.9	90.9	101.0, 93.9	97.5
45	94.7, 92.9	93.8	98.0, 84.8	91.4	93.9, 92.9	93.4
60	95.6, 98.2	96.9	91.9, 86.9	89.4	102.0, 90.9	96.5
90	94.7, 95.6	95.2 (mean 98.0)	96.0, 82.8	89.4 (mean 92.4)	98.0, 90.9	94.5 (mean 96.4)
Dark controls						
	pH 5[1]		pH 7[2]		pH 9[3]	
0	100.0		100.0		100.0	
10	102.7		84.8		83.8	
20	100.9		88.9		91.9	
30	100.9		85.9		88.9	
45	97.3		83.8		83.8	
60	103.5		85.9		83.8	
90	94.7		78.8		80.8	

Values are percentages of initial concentrations (nominal concentration 1 mg/L, actual initial concentrations were 1.13, 0.99 and 0.99 mg/L at pH values 5, 7 and 9 respectively).

The overall range of total recovery from the individual exposed samples was 82.8% to 106.2% (overall average 98.0, 92.4 and 96.4% at pH values of 5, 7 and 9 respectively). The overall range of total recovery from the individual dark control samples was 78.8% to 102.7% (overall average 100.0, 86.9 and 87.6% at pH values of 5, 7 and 9 respectively).

1 Measured pH values 4.95 at 0 mins, 4.95 at 90 mins.
2 Measured pH values 7.25 at 0 mins, 7.20 at 90 mins.
3 Measured pH values 8.85 at 0 mins, 8.85 at 90 mins.
4 For the dark controls, single samples were taken for analysis from each pH at each sampling interval.

Table A 7.1.1.1.2-5: Concentration of difethialone in sterile aqueous buffer exposed to natural sunlight

Incubation time (mins)	Concentration of difethialone in buffer solution (% AR)					
	pH 5		pH 7		pH 9	
	Measured	Mean	Measured	Mean	Measured	Mean
SS 1 (solvent system 1)						
0	92.5	92.5	90.4	90.4	91.1	91.1
10	87.2, 87.7	87.5	76.4, 63.2	69.8	68.8, 54.8	61.8
20	72.7, 88.6	80.7	67.9, 75.4	71.7	45.7, 50.6	48.2
30	67.9, 81.6	74.8	62.8, 60.6	61.7	47.0, 52.6	49.8
45	46.1, 57.4	51.8	46.2, 41.7	44.0	37.5, 39.4	38.5
60	44.8, 48.6	46.7	40.0, 34.5	37.3	34.2, 38.0	36.1
90	33.4, 38.9	36.2	39.8, 38.7	39.3	30.0, 31.5	30.8
SS 2 (solvent system 2)						
0	87.4	87.4	97.3	97.3	88.5	88.5
10	91.4, 87.3	89.4	92.9, 79.7	86.3	80.4, 83.0	81.7
20	92.2, 93.9	93.1	91.0, 88.9	90.0	81.4, 82.4	81.9
30	91.8, 87.9	89.9	82.3, 80.6	81.5	76.6, 82.3	79.5
45	82.4, 82.0	82.2	81.2, 84.6	82.9	80.0, 73.6	76.8
60	83.5, 82.6	83.1	70.0, 76.4	73.2	71.6, 78.1	74.9
90	75.6, 76.4	76.0	76.2, 79.9	78.1	72.4, 73.5	73.0

Values are expressed as a percentage of the initial concentration.

Table A 7.1.1.1.2-6: Concentration of difethialone in sterile aqueous buffer in dark controls

Incubation time (mins)	Concentration of difethialone in buffer solution (% AR)					
	pH 5		pH 7		pH 9	
	SS 1	SS 2	SS 1	SS 2	SS 1	SS 2
0	92.5	87.4	90.4	97.3	91.1	88.5
10	85.4	97.4	83.4	93.2	84.7	93.3
20	81.7	92.3	81.0	93.5	85.6	96.2
30	83.9	94.0	85.3	95.5	88.6	95.0
45	86.2	91.4	73.8	93.0	86.6	93.0
60	86.9	94.5	79.2	81.1	80.9	92.5
90	86.4	87.0	74.0	94.9	77.3	96.1

Values are expressed as a percentage of the initial concentration.

Figure A 7.1.1.1.2-1: Re-calculation of DT_{50} and DT_{90} values for photolysis of difethialone in sterile aqueous buffer (pH 5)

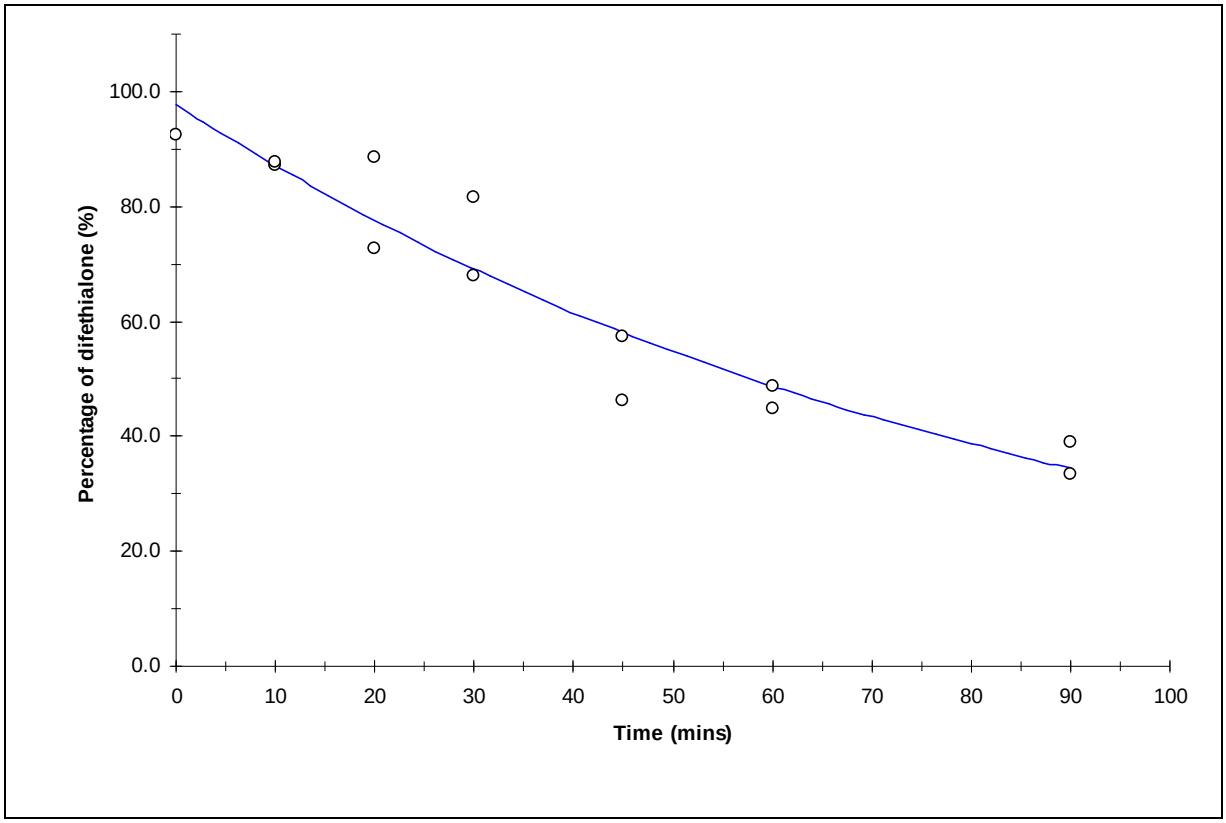


Figure A 7.1.1.1.2-2: Re-calculation of DT_{50} and DT_{90} values for photolysis of difethialone in sterile aqueous buffer (pH 7)

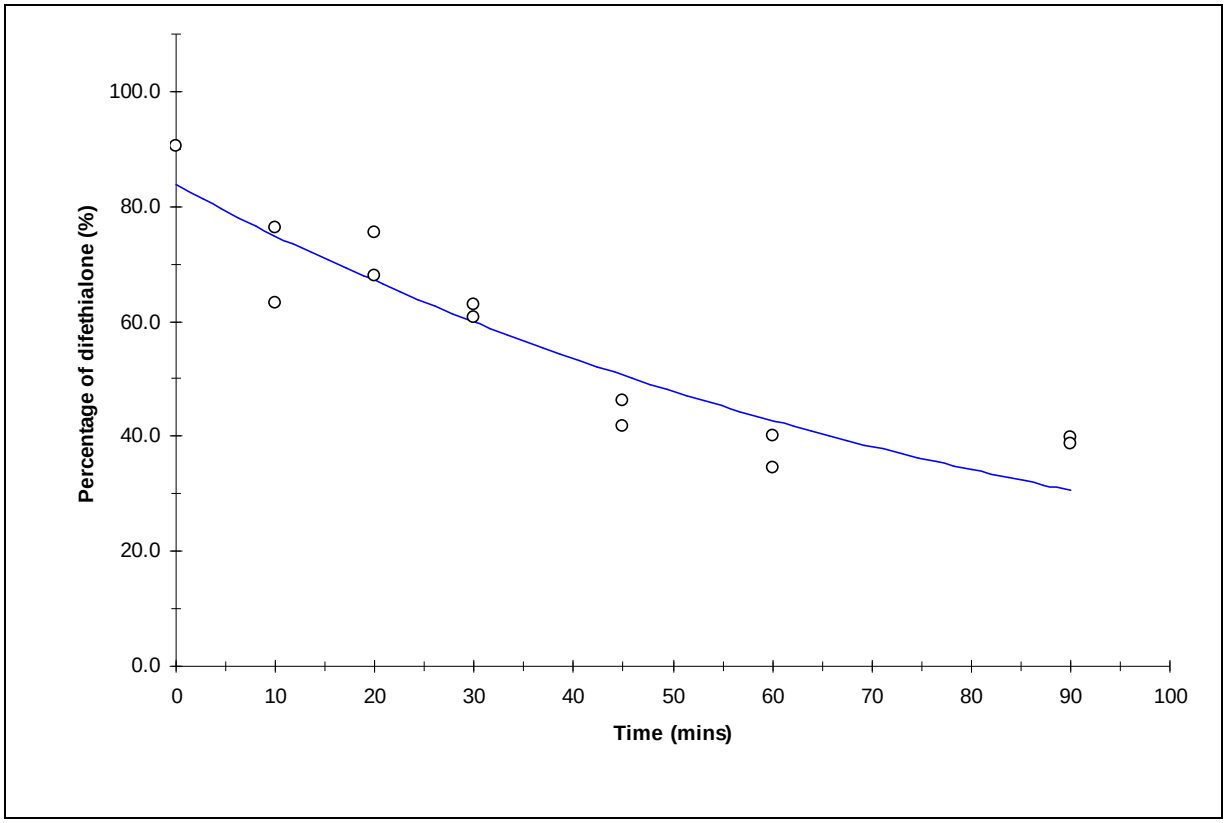


Figure A 7.1.1.1.2-3: Re-calculation of DT_{50} and DT_{90} values for photolysis of difethialone in sterile aqueous buffer (pH 9)

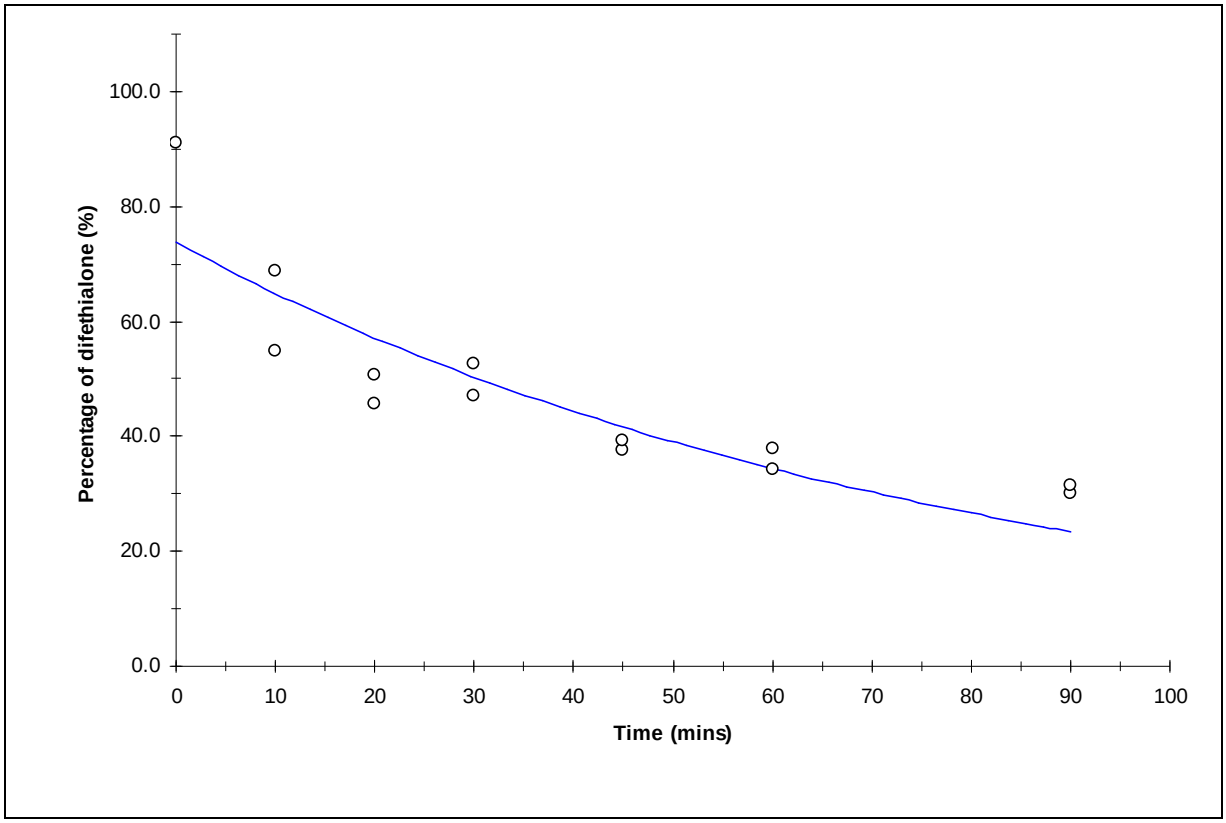


Table A 7.1.1.1.2-7: First order DT_{50} and DT_{90} values for the rate of photolysis of difethialone in sterile aqueous buffer

Buffer	Data range (mins)	$DT_{50(lab)}$ (mins)	$DT_{90(lab)}$ (mins)	Regression parameters		
				C_0 (% AR)	k (min^{-1})	R^2
pH 5	0 to 90 [1]	59.7	198 [2]	97.83	0.01161	0.91
pH 7	0 to 90 [1]	61.9	206 [2]	83.82	0.01120	0.85
pH 9	0 to 90 [1]	54.5 (overall average 58.7 mins)	181 [2]	73.69	0.01271	0.78

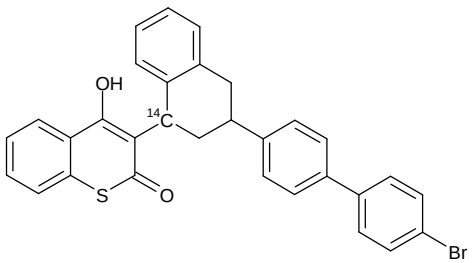
[1] All data points were used.

[2] DT_{50} (or DT_{90}) value was not demonstrated experimentally, result obtained by extrapolation.

Table A 7.1.1.1.2-8: Concentration of photolysis products in sterile aqueous buffer exposed to natural sunlight

Incubation time (mins)	Concentration of photolysis component (% AR)			
	Origin		Rf 0.8	
	Measured	Mean	Measured	Mean
pH 5 buffer				
0	6.4	6.4	0.6	0.6
10	6.7, 8.5	7.6	3.2, 1.4	2.3
20	13.0, 10.1	11.6	7.8, 1.2	4.5
30	14.5, 13.1	13.8	10.1, 5.2	7.7
45	20.7, 20.5	20.6	19.2, 11.7	15.5
60	27.1, 28.2	27.7	12.3, 9.6	11.0
90	31.0, 28.7	29.9	24.6, 21.7	23.2
pH 7 buffer				
0	7.0	7.0	2.2	2.2
10	16.2, 14.9	15.6	4.0, 9.2	6.6
20	18.0, 19.7	18.9	6.2, 4.6	5.4
30	15.8, 16.8	16.3	10.2, 12.8	11.5
45	23.4, 24.8	24.1	14.6, 15.7	15.2
60	32.9, 31.9	32.4	10.9, 13.7	12.3
90	30.6, 28.2	29.4	16.0, 20.5	18.3
pH 9 buffer				
0	6.6	6.6	2.2	2.2
10	31.2, 25.0	28.1	0.0, 12.2	6.1
20	25.9, 25.3	25.6	13.1, 13.8	13.5
30	26.0, 22.6	24.3	14.2, 9.4	11.8
45	36.6, 40.3	38.5	8.9, 13.0	11.0
60	40.5, 38.9	39.7	8.2, 7.2	7.7
90	37.4, 40.6	39.0	20.9, 17.7	19.3

Values are expressed as a percentage of the initial concentration.

Section A 7.1.1.1.2 **Annex Point IIA VII.7.6.2.2**		**Phototransformation in water including identity of transformation products**	
		6 REFERENCE	**Official use only**
6.1	**Reference**	Xxxx, X., Xxxxxxxxxxx, XX. and Xxxxxxx, X., XXXX, Artificial sunlight photodegradation of [^{14}C]-difethialone in buffered aqueous solution. Xxxxxxxxx Xxxxxxxxxx, laboratory report no. XXXXX, XX Xxxxxxxx XXXX (unpublished). Section no.: A 7.1.1.1.2-02.	
6.2	**Data protection**	Yes.	
6.2.1	Data owner	LiphaTech S.A.S.	
6.2.2	Companies with letter of access	None.	
6.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		7 GUIDELINES AND QUALITY ASSURANCE	
7.1	**Guideline study**	Yes. The study was performed to SETAC Guidelines.	
7.2	**GLP**	Yes.	
7.3	**Deviations**	None. The study was conducted in the recommended guideline (SETAC, OPPTS 835.2210).	
		8 MATERIALS AND METHODS	
8.1	**Test material**	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one.	
8.1.1	Lot/Batch number	Batch XXX XXXXX.	
8.1.2	Specification	Specific activity 1.92 GBq/mmol.	
8.1.3	Purity	RCP (radiochemical purity) XX.X% by HPLC.	
8.1.4	Radiolabelling	Position of radiolabel given below: 	
8.1.5	Further relevant	None.	

Section A 7.1.1.1.2 Annex Point IIA VII.7.6.2.2		Phototransformation in water including identity of transformation products	
	properties		
8.2	**Test material (non-radiolabelled)**	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one.	
8.2.1	Lot/Batch number	Batch no. XXXX XXXXXX.	
8.2.2	Specification	No further details.	
8.2.3	Purity	XX.X%.	
8.2.4	Further relevant properties	Not applicable.	
8.3	**Reference substances**	Analytical reference standards of LM2963 (batch no. XXXXXX) and LM3541(batch no. XXXXXXX).	
8.4	**Testing procedure**	The rate of photolysis of difethialone in aqueous solution, at a pH value of 7, was investigated under artificial sunlight. The quantum yield of difethialone was also determined.	
8.4.1	Test system	Neutral buffer solution was prepared using distilled, deionised water as described in Table A 7.1.1.1.2-8. All buffer solutions were sterilised by filtration (0.22 µm). All glassware were sterilised by autoclaving. The treatment and incubation of the test solutions is summarised in Table A 7.1.1.1.2-9. Further details of the test system and equipment used is provided in Table A 7.1.1.1.2-10.	
8.4.2	Properties of light source	An artificial light source was used, see Table A 7.1.1.1.2-10. The light source utilised a xenon light source, the spectral distributin of the light source was shown to resemble natural sunlight.	
8.4.3	Determination of irradiance	See Table A 7.1.1.1.2-10. The incident irradiance on the sample solutions was determined using a radiometer.	
8.4.4	Temperature	Test samples were incubated in a water bath (20 ± 3°C).	
8.4.5	pH	The study was conducted at a pH value of 7.	
8.4.6	Duration of the test	The definitive phase of the study was conducted over 2 hours continuous exposure to artificial sunlight.	
8.4.7	Number of replicates	Duplicate exposed samples at each sampling interval. A single dark control sample was taken at 48 hours.	
8.4.8	Sampling	Samples were taken for analysis at intervals of 0, 20, 40 minutes and 1, 2, 4, 24 and 48 hours. At each sampling interval the level of radioactivity in the buffer solutions was quantified by removing an aliquot (1 mL) for LSC. The remaining sample was decanted from the incubation vessel into acetonitrile (10 mL) to reduce any further potential losses to glassware via absorption. The incubation vessel was washed with additional acetonitrile (5 mL) to recover any radioactivity absorbed to the glassware during incubation. Aqueous samples were analysed by HPLC using the methods described	

Section A 7.1.1.1.2 Annex Point IIA VII.7.6.2.2		Phototransformation in water including identity of transformation products	
		in Section 3.4.9.	
8.4.9	Analytical methods	For routine chromatographic analysis the buffer solutions were analysed by HPLC using a reverse phase system, see details in Table A 7.1.1.1.2-10. Recovery of radioactivty during HPLC sample analysis was 88 to 104%. TLC analysis was conducted using silica gel plates developed in methanol/ acetic acid (9:1 v/v). Non radiolabelled reference standards were visualised using UV light (254 nm). Radioactive regions were quantified using a Phosphor imager. The RCP determinations were conducted using the HPLC conditions.	
8.5	**Transformation products**		
8.5.1	Method of analysis for transformation products	The levels of difethialone and corresponding degradation products were monitored using the chromatographic systems described in Section 3.4.9.	
8.6	**Quantum yield determination**	A p-nitroanisole (PNA)/ pyridine actinometer was used to determine the quantum yield of difethialone.	
		9 RESULTS	
9.1	**Screening test**	Some preliminary tests were performed using the same methodologies as described above, however these were used solely as range finders (i.e. to determine the required exposure duration etc.) and the results are not described in detail in the study report.	
9.2	**Photolysis data**		
9.2.1	Recovery of applied radioactivity, mass balance	The recovery of applied radioactivity from the exposed samples and dark controls is summarised in Table A 7.1.1.12.-11. The amount of applied radioactivity recovered from the exposed samples ranged from 86.88 to 105.15% (overall average 97.39%) indicating a complete mass balance. The majority of the applied radioactivity was recovered as part of the incubate at all sampling intervals (> 70%), however, significant amounts were recovered as vessel washes following absorption to glass surfaces. The amount of radioactivity recovered as vessel washes was highly variable, even between replicate samples. Minimal amounts were recovered as evolved volatile components (< 1% AR). The recovery of applied radioactivity from the dark control sample ranged from 101.67 to 103.37% AR (mean 102.52% AR). Approximately 70% of the radioactivity was recovered in the incubate, the remaining 30% was recovered as a vessel wash.	

Section A 7.1.1.1.2 **Annex Point IIA VII.7.6.2.2**		**Phototransformation in water including identity of transformation products**	
9.2.2	Chromatographic analysis	The chromatographic analysis of the incubates and the vessel washes is summarised in Table A 7.1.1.1.2-12. The levels of difethialone detected in the incubates declined rapidly from *ca* 78% initially to *ca* 2.5% after 2 hours exposure. After 4 hours exposure, the levels of difethialone remaining in the water were not detectable. Exposure of difethialone to artificial light led to the formation of numerous photo-degradation products (i.e. at least 10). The combined total level of all degradation products comprised *ca* 87% after 48 hours. Three degradation products (unknowns A, B and C) were clearly resolved but were not identified further. After 48 hours only components unknown A and unknown B were present at levels > 10% AR, however, due to the short retention times of these components it is possible that these components were not single entities and were actually comprised of many individual components. The radioactivity remaining in the dark control sample after 48 hours was shown to be mostly difethialone (mean 69%) with most of the remaining material recovered in the vessel wash as difethialone (*ca* 29%). Some degradation was observed in the dark control sample after 48 hours, however this was minimal (*ca* 4.5%) and indicated that in the dark, in aqueous solution difethialone was reasonably stable.	**x**
9.2.3	Photolysis rate constant, k^c_p	The photolysis of difethialone under artificial sunlight was rapid. After 2 hours exposure the amount of difethialone comprised *ca* 2.5%. The photo-degradation of difethialone is represented graphically in Figure A 7.1.1.1.2-4 and summarised in Table A 7.1.1.1.2-13. The photo-degradation of difethialone in the exposed incubate gave a good correlation to pseudo first order kinetics (R^2 = 0.959). The degradation rate was not corrected for the minimal degradation observed in the dark control sample. Furthermore, the determined photo-degradation rate only considered the radioactivity recovered in the incubate itself and excluded the material recovered in the vessel washes. The best fit rate constant for photo-degradation of difethialone in aqueous solution was determined to be 0.02975 min^{-1}, the corresponding DT_{50} value was 23.4 minutes (DT_{90} value 77.4 minutes).	
9.2.4	Kinetic order	The photolysis of difethialone, under artificial sunlight, gave a good correlation to pseudo first order kinetics (R^2 = 0.959).	
9.2.5	Reaction quantum yield (ϕ^c_E)	The quantum yield (ϕ^c_E) for difethialone was determined to be 6.183 x 10^{-3} mol/photon.	
9.2.6	Calculation of DT_{50} values at latitudes of 40°N and 50°N	The DT_{50} value for difethialone in the summer at latitudes 40°N and 50°N were determined to be 10.02 and 10.35 minutes respectively. In the winter the DT_{50} values were 30.24 and 60.05 minutes respectively.	
		10 APPLICANT'S SUMMARY AND CONCLUSION	
10.1	**Materials and methods**	The rate of photolysis of difethialone in aqueous solution, at a pH value of 7, was investigated under artificial sunlight. The GLP study was conducted to SETAC Guidelines in 2003.	

Section A 7.1.1.1.2 **Annex Point IIA VII.7.6.2.2**	**Phototransformation in water including identity of transformation products**	
10.2 Results and discussion	The recovery of applied radioactivity ranged from 86.88 to 105.15% (overall average 97%). Variable amounts of difethialone were absorbed to glassware during the incubation period. No evolved volatile components were observed. Photolysis of difethialone was extensive and rapid. Photolysis gave a good correlation to pseudo first order kinetics.	
10.2.1 k^c_p	The rate constant was 0.02975 min^{-1}.	
10.2.2 $t_{1/2E}$	The DT_{50} value was 23.4 mins (corresponding DT_{90} value 77.4 mins).	
10.2.3 ϕ^c_E	The quantum yield (Φ) for difethialone was determined to be 6.183 x 10^{-3} mol/photon.	
10.2.4 Calculation of DT_{50} values at latitudes of 40°N and 50°N	The DT_{50} value for difethialone in the summer at latitudes 40°N and 50°N were determined to be 10.02 and 10.35 minutes respectively. In the winter the DT_{50} values were 30.24 and 60.05 minutes respectively.	
10.3 Conclusion	Difethialone is rapidly and extensively photo-degraded in water with an estimated DT_{50} value of 23.4 minutes. Photo-degradation was extensive, after 2 hours exposure numerous polar degradation products were formed which comprised *ca* 80% of applied radioactivity. The quantum yield (Φ) for difethialone was determined to be 6.183 x 10^{-3} mol/photon.	**x**
10.3.1 Reliability	2	
10.3.2 Deficiencies	Yes. The study contained some deficiencies when compared to modern day standards. These deficiencies are discussed in more detail where appropriate under the relevant headings above and are not considered to have adversely affected the quality of the results. In addition, the study made no attempt to identify the photolysis components formed in significant quantities (i.e. > 10% AR). As the study is conducted for classification purposes only (i.e. actual use of the biocidal products will not result in exposure to aquatic systems) the identity of the photolysis components is not considered relevant.	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	27 December 2004; revised 31st October 2006	
Materials and Methods	Agree with applicant's version	
Results and discussion	Agree with applicant's summary and conclusion. **Comments (4.2.2.)** Transformation products accounting for ca 87 % AR were observed but not identified as required according to Annex Point IIA VII.7.6.2.2.	

Section A 7.1.1.1.2 **Annex Point IIA VII.7.6.2.2**	**Phototransformation in water including identity of transformation products**	
Conclusion	**Comment (5.3):** Difethialone is rapidly and extensively photo-degraded in water with an estimated DT_{50} value of 23.4 minutes. Photo-degradation was extensive, after 2 hours exposure numerous polar degradation products were formed which comprised *ca* 80% of applied radioactivity. The transformation products were not identified. After 48 hours exposure two of the unidentified components were present at levels > 10 % (66 and 20 % AR respectively). These components are more polar than difethialone. Due to the short retention times of these components in the chromatogram (2.2 and 3.3 min) it is possible that these components were not single entities but actually comprised of many individual substances. According to the TNsG any metabolites or other degradation products that at any sampling time during the studies account for more than 10% of the active substance added should be identified and their degradation rates should be studied. However, as the water compartment is not the main compartment of risk no further testing is required. Moreover, the high adsorption tendency of difethialone to organic matter further reduces difethialone concentration in water bodies. Photodegradation in the environment only takes place in the upper layers of surface water. Laboratory results, carried out with distilled or tap water, do not represent environmental conditions, where surface water is normally deeper and muddier. Considering the total water body photodegradation is therefore not considered to be a relevant process for difethialone dissipation in the environment and this justifies also why further testing is not considered necessary. The quantum yield (Φ) for difethialone was determined to be 6.183×10^{-3} mol/photon. More information on the metabolites which have been formed and a potential photodegradation pathway are presented in a position paper received from the applicant. This position paper is presented as an attachment to this study summary.	
Reliability	2	
Acceptability	Acceptable with the restrictions noted above	
Remarks		

Table A 7.1.1.1.2-8: Type and composition of buffer solution

pH	Type of buffer (final molarity)	Composition
7	Phosphate buffer (0.007M)	0.067M Sodium phosphate and 0.067M dipotassium phosphate, mixed in the ratio 30:61 v/v and diluted tenfold. Adjusted to pH 7 by addition of phosphoric acid.

Table A 7.1.1.1.2-9: Description of test solution and controls

Criteria	Details
Purity of water	Distilled, deionised.
Preparation of test medium	Test substance (8.6 µg) dissolved in acetone (200 µL) was added to buffer solution (*ca* 20 mL) in a quartz glass vial. Multiple samples were prepared for exposure to the artificial light source (duplicate samples at eight sampling intervals). One further sample was prepared and incubated in the dark for use as a dark control.
Test concentrations (mg a.i./L)	0.4 mg/l
Temperature (°C)	20 ± 3°C.
Controls	Dark control samples were similarly prepared.
Identity and concentration of co-solvent	Acetonitrile and acetone 1% v/v.
Replicates	Duplicate exposed samples at each sampling interval. Duplicate dark control samples were taken for analysis at 48 hours only.

Table A 7.1.1.1.2-10: Description of test system

Criteria	Details
Glassware	Sample solutions were exposed to the artificial light source in quartz glass vials.
Other equipment	Liquid scintillation counter. HPLC (Hewlett Packard 1050 / Agilent 1100) with flow through radio-detector (Packard 150TR) and UV detector (254 nm). TLC (Fuji FLA 5000 Phosphor Imager).
Method of sterilisation	The buffer solutions were sterilised by filtration (0.22 µm). The glassware was sterilised by autoclaving.
Properties of artificial light source:	Heraeus Suntest CPS (Heraeus Equipment Ltd.) using a xenon irradiation source fitted with filters to remove light of wavelength < 290 nm and > 800 nm. Irradiation intensity (*ca* 456 W/m^2) was measured using a handheld radiometer (Heraeus Radialux Original Hanau monitor).

Table A 7.1.1.1.2-11: Recovery of applied radioactivity from sterile aqueous buffer

Incubation time	Recovery of applied radioactivity (% AR)				
	Incubation vessel		Volatile components		Total
	Incubate	Vessel wash	Carbon dioxide	Other volatiles	
0	93.08, 66.20 (79.64)	7.53, 31.21 (19.37)	n.a	n.a	100.61, 97.41 (99.01)
20 min	70.02, 84.75 (77.39)	27.72, 16.69 (22.21)	0.02	0.01	97.77, 101.47 (99.62)
40 min	89.16, 73.25 (81.21)	12.26, 25.89 (19.08)	0.01	0.02	101.45, 99.17 (100.31)
1 hour	74.19, 77.32 (77.32)	25.01, 17.25 (21.13)	0.01	0.03	99.24, 97.73 (98.49)
2 hours	83.72, 85.08 (84.40)	14.34, 14.01 (14.18)	0.01	0.33	98.40, 99.43 (98.92)
4 hours	84.53, 86.68 (85.61)	9.52, 10.85 (10.19)	0.01	0.73	94.79, 98.27 (96.53)
24 hours	81.57, 88.99 (85.28)	5.20, 2.54 (3.87)	0.02	0.09	86.88, 91.64 ()89.26
48 hours	87.16, 87.56 (87.46)	1.60, 17.34 (9.47)	0.01	0.04	88.81, 105.15 (96.98)
48 hours (dark control)	69.16, 75.05 (72.11)	32.50, 28.30 (30.40)	0.01	0.01	101.67, 103.37 (102.52)

Values are percentages of applied radioactivity (% AR).

The overall range of total recovery from the individual exposed samples was 86.88% to 105.15% (overall average 97.39%).

Table A 7.1.1.1.2-12: Concentration of difethialone in sterile aqueous buffer exposed to artificial sunlight

Incubation time	Concentration of difethialone in buffer solution (% AR)					
	Difethialone	Unk A	Unk B	Unk C	Other unknowns	Total
Incubates						
0	91.04, 64.64 (77.84)	2.04, 1.56 (1.80)	n.d, n.d (n.d)	n.d, n.d (n.d)	n.d, n.d (n.d)	93.08, 66.20 (79.64)
20 min	33.75, 36.00 (34.88)	7.17, 9.38 (8.28)	5.38, 3.94 (4.66)	2.23, 2.16 (2.20)	21.50, 33.26 (27.38)	70.02, 84.75 (77.39)
40 min	30.06, 15.88 (22.97)	12.30, 8.30 (10.30)	8.43, 10.38 (9.41)	9.71, 4.67 (7.19)	28.66, 34.02 (31.34)	89.16, 73.25 (81.21)
1 hour	7.01, 9.05 (8.03)	9.89, 11.52 (10.71)	14.69, 17.56 (16.13)	3.56, 4.59 (4.08)	39.04, 37.72 (38.38)	74.19, 80.44 (77.32)
2 hours	1.94, 3.08 (2.51)	9.56, 9.89 (9.73)	29.87, 30.21 (30.04)	11.43, 16.49 (13.96)	30.92, 25.40 (28.16)	83.72, 85.08 (84.40)
4 hours	n.d, n.d (n.d)	2.65, 5.50 (4.08)	44.52, 44.06 (44.29)	6.00, 5.32 (5.66)	31.35, 31.80 (31.58)	84.53, 86.68 (85.61)
24 hours	n.d, n.d (n.d)	n.d, n.d (n.d)	59.71, 65.79 (62.75)	19.85, 17.82 (18.84)	2.01, 5.38 (3.70)	81.57, 88.99 (85.28)
48 hours	n.d, n.d (n.d)	n.d, n.d (n.d)	64.70, 67.00 (65.85)	19.23, 20.76 (20.00)	3.23, n.d (1.62)	87.16, 87.16 (87.46)
48 hours (dark controls)	64.55, 73.65 (69.10)	n.d, n.d (n.d)	n.d, n.d (n.d)	n.d, n.d (n.d)	4.61, 1.40 (3.01)	69.16, 75.05 (72.11)
Vessel washes						
0	7.53, 29.85 (18.69)	n.d, 1.36 (0.68)	n.d, n.d (n.d)	n.d, n.d (n.d)	n.d, n.d (n.d)	7.53, 31.21 (19.37)
1 hour	6.13, 2.65 (4.39)	4.83, 3.75 (4.29)	2.55, 1.20 (1.88)	1.48, 0.93 (1.21)	10.03, 8.71 (9.37)	25.01, 17.25 (21.13)
48 hours	n.p 16.71 (16.71)	n.p, 0.64 (0.64)	n.p, n.d (n.d)	n.p, n.d (n.d)	n.p, n.d (n.d)	n.p, 17.34 (17.34)
48 hours (dark controls)	31.03, 26.74 (28.89)	n.d, n.d (n.d)	n.d, n.d (n.d)	n.d, n.d (n.d)	1.47, 1.578 (1.52)	32.50, 28.30 (30.40)

Values are expressed as a percentage of the applied radioactivity.

n.p – not performed.
n.d – not detected.

Figure A 7.1.1.1.2-4: DT_{50} and DT_{90} values for photolysis of difethialone in sterile aqueous buffer (pH 7)

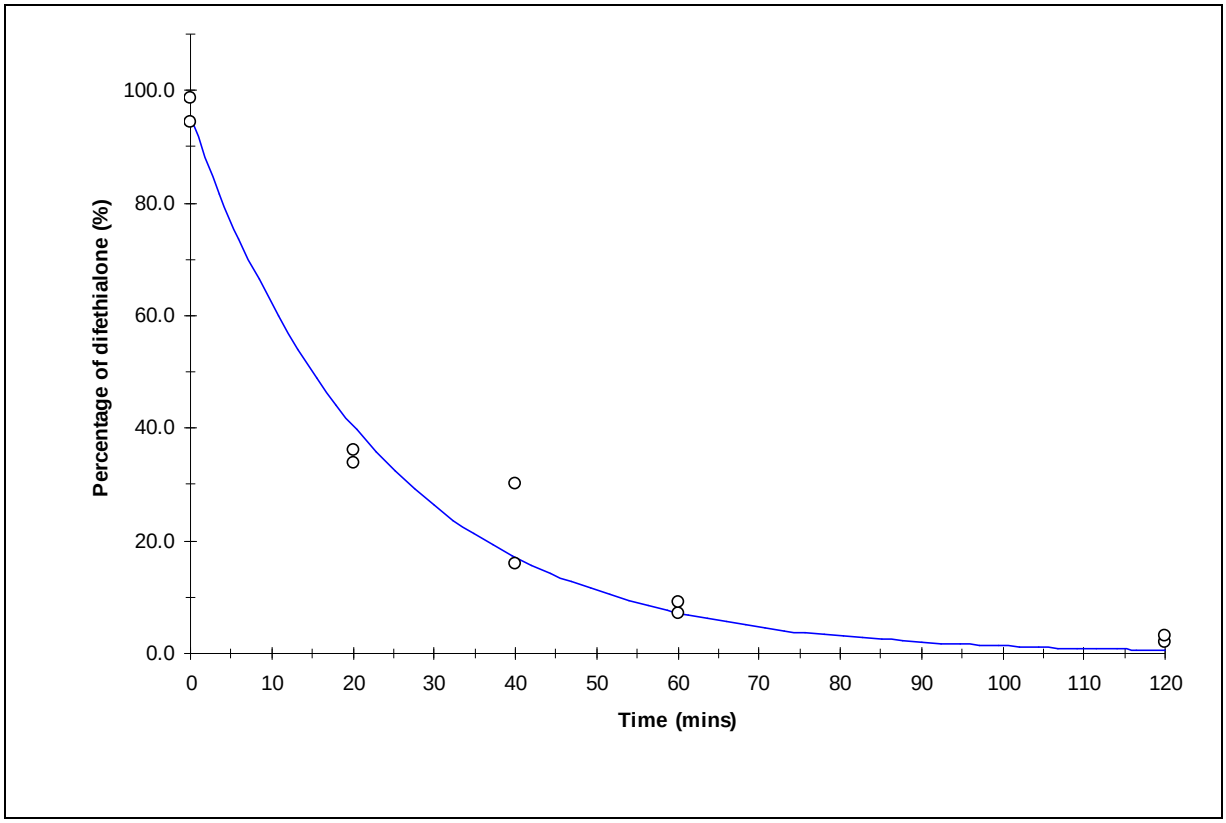


Table A 7.1.1.1.2-13: First order DT_{50} and DT_{90} values for the rate of photolysis of difethialone in sterile aqueous buffer

Buffer	Data range (mins)	$DT_{50(lab)}$ (mins)	$DT_{90(lab)}$ (mins)	Regression parameters		
				C_0 (% AR)	k (min^{-1})	R^2
pH 7	0 to 120 [1]	23.4	77.4	72.00	0.02975	0.9594

[1] Data points from 0 hours to 2 hours (120 minutes) were used (i.e. data points between 4 and 48 hours were not used).

[2] DT_{50} (or DT_{90}) value was not demonstrated experimentally, result obtained by extrapolation.

Attachment to Study Summary III A 7.1.1.1.2-02

Difethialone

Review of existing active substance

Dossier submitted under 98/8/EC

Position paper on aqueous photolysis photodegradation products

LiphaTech S.A.S.

May 2006

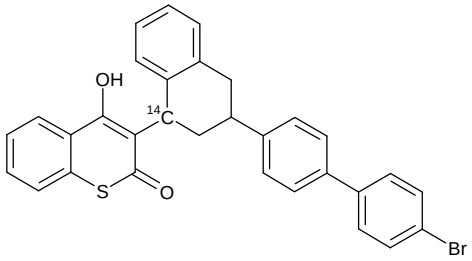
Introduction

This position paper was prepared in response to a request at the Technical meeting in April discussing Difethialone.

Aqueous photolysis of difethialone

A study was conducted (Xxxxx, X., Xxxxxxxxxxxx, XX. and Xxxxxxxxx, X., XXXX, ref IIIA 7.1.1.1.2-02) to investigate the aqueous photolysis of difethialone in buffered solution (pH 7) under artificial sunlight. The study was conducted to the SETAC guidelines.

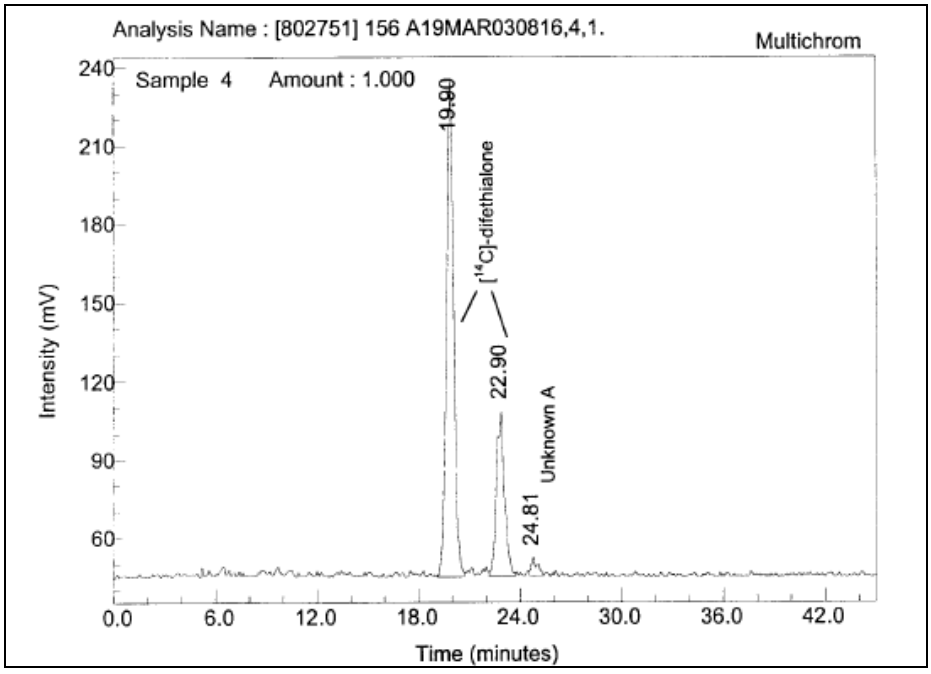
The structure of difethialone is given below (the position of the 14C radiolabel used in the aqueous photolysis study is shown):



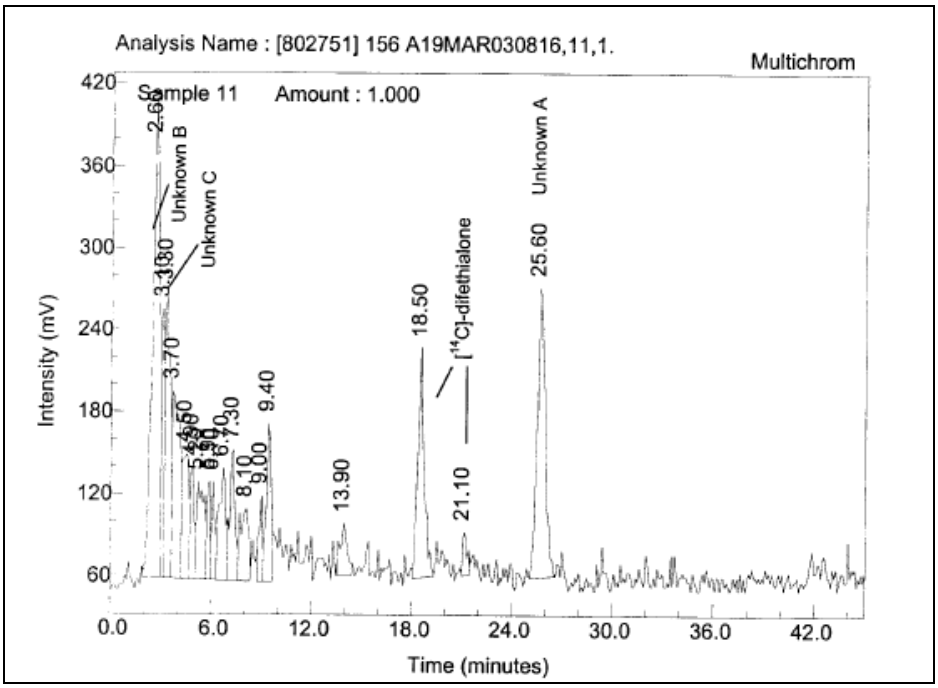
Under the study conditions a solution of difethialone (0.4 mg/L) was exposed to a Xenon light source (wavelength 290 to 800 nm) for a duration of 48 hours to resemble exposure to natural sunlight. Samples were removed for analysis at intervals of 0, 20, 40 minutes and 1, 2, 4, 24 and 48 hours.

The following reverse phase HPLC chromatographs depict the profile of the components observed:

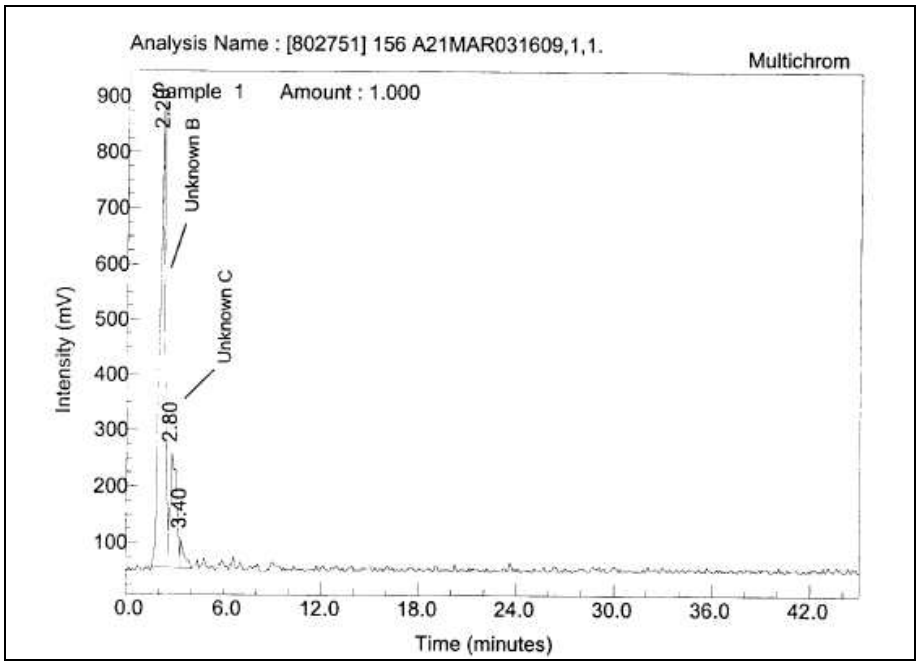
a) Start of study:



b) 1 hour exposure:



b) 48 hour exposure:



The chromatographs show, a) at the start of the study, the two peaks corresponding to the isomers of difethialone, b) the peaks relating to difethialone were diminished after 1 hr exposure and the formation of multiple photodegradation products, and c) the complete photolysis of difethialone after 48 hrs exposure. Photodegradation of difethialone in aqueous solution led to the formation of multiple components.

Due to the extensive photolysis observed, a comprehensive quantitative description and resolution of all the components observed was not feasible. However, a number of distinct "regions of interest" were consistently observed and quantifiable, these were named Unknown A (a component with retention time ca 25 mins), Unknown B (a component with retention time ca 2.6 mins) and Unknown C (a component with retention time ca 3.3 mins). It is worth noting at this stage that the components Unknown B and Unknown C, due to the low retention observed in the analysis conditions, could possible not correspond to single components.

A full profile of the chromatographic analysis is given in the table below:

Table 1: Concentration of difethialone in sterile aqueous buffer exposed to artificial sunlight

Incubation time	Concentration of difethialone in buffer solution (% AR)					
	Difethialone	Unk A	Unk B	Unk C	Other unknowns	Total
Incubates						
0	77.84	1.80	n.d	n.d	n.d	79.64
20 min	34.88	8.28	4.66	2.20	27.38	77.39
40 min	22.97	10.30	9.41	7.19	31.34	81.21
1 hour	8.03	10.71	16.13	4.08	38.38	77.32
2 hours	2.51	9.73	30.04	13.96	28.16	84.40
4 hours	n.d	4.08	44.29	5.66	31.58	85.61
24 hours	n.d	n.d	62.75	18.84	3.70	85.28
48 hours	n.d	n.d	65.85	20.00	1.62	87.46
48 hours (dark controls)	69.10	n.d	n.d	n.d	3.01	72.11
Vessel washes						
0	18.69	0.68	n.d	n.d	n.d	19.37
1 hour	4.39	4.29	1.88	1.21	9.37	21.13
48 hours	16.71	0.64	n.d	n.d	n.d	17.34
48 hours (dark controls)	28.89	n.d	n.d	n.d	1.52	30.40

Values are expressed as a percentage of the applied radioactivity.
n.p – not performed.
n.d – not detected.

Two of the components observed, Unknown B and Unknown C, were formed in ever increasing amounts over the study duration and comprised 66 and 20% of applied radioactivity, respectively, after 48 hours. These components were formed in large amounts at times when the amount of parent difethialone remaining was negligible, indicating that these photodegradation products were formed via unidentified intermediates. No further photodegradation of these components was apparent and therefore it is assumed that they were stable under these conditions (the study showed that the photodegradation did not ultimately lead to the formation of carbon dioxide). These components were therefore assumed to be terminal photolysis products and can be seen to be relatively polar in nature i.e. short retention times). Furthermore use of the radiolabelled active substance means that any photodegradation products observed contained the specific radiolabelled carbon.

The component Unknown A, although observed in the 0 hr sample and to some degree in the pre-treatment purity checks, was observed at significantly greater amounts after 1 hrs exposure and can therefore be considered to be a genuine photodegradation product. The Unknown A component was initially formed in appreciable quantities very early (i.e. 20 to 40 mins), however, once the levels of parent difethialone remaining had diminished, the rate of formation of was negligible. It can therefore be assumed that Unknown A was formed directly from the parent difethialone and that, as the rate of decline of Unknown A was somewhat slower than the rate of formation of the components Unknowns B and C, it is assumed that these components were part of competing pathways. It is also observed that the retention time of the component Unknown A relative to parent difethialone, indicates that it is more non-polar.

Further discussion on the likely identity of the photodegradation components is purely speculative. Photolysis reactions are somewhat less predictable then those associated with microbial or abiotic (i.e.

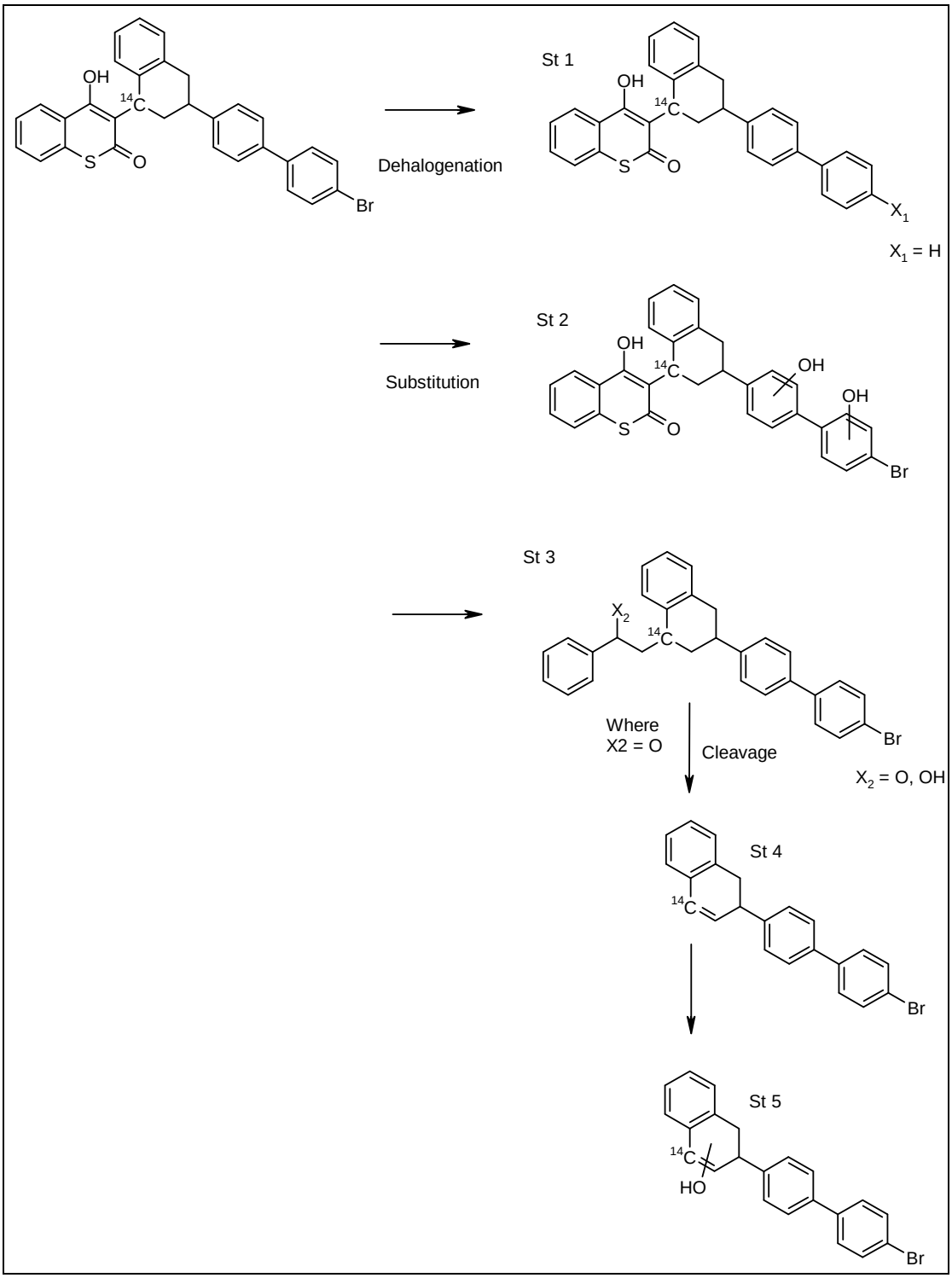
hydrolysis) degradation. Potential photolysis reactions include radical formation and subsequent rearrangement/cyclisation, photo-reduction (aldehydes and ketones), dealkylation of substituted compounds, dehalogenation, substitution etc.

Considering the functional groups present in the difethialone molecule any potential initial steps appear limited. Potential photodegradation pathways are depicted in Figure 1:

Taking into account the observations described above, the component Unknown A could be associated with structure St 1 and/or structure St 4 or a combination. This would be consistent with a component that was initially formed quite quickly and of a non-polar nature (relative to the parent active substance), which then seemed to be photodegraded relatively slowly. The components Unknown B and C, being polar in nature, are potentially associated with the structures St 2 and St 5, where the possibilities for substitution reactions had been exhausted. This would be consistent with the hypothesis that these were terminal metabolites of a relatively polar nature.

Obviously, many other potential photodegradation products are possible (radical formation and any subsequent rearrangement and/or cyclisation has not been considered above to avoid further complication of the tentative structures proposed), however the observations indicate that difethialone is extensively photodegraded into multiple components of a much more polar nature than the parent active substance molecule, which would indicate that the active moiety as no longer intact.

Figure 1: Potential photodegradation pathways of difethialone



Section A 7.1.1.2.1 **Annex Point IIA VII.7.6.1.1**		**Biodegradability (ready)**	
		11 REFERENCE	**Official use only**
11.1	**Reference**	Xxxxxx, X. and Xxxxxxxx, XX., XXXXx, Difethialone: Determination of 28 day ready biodegradability (CO_2 headspace test). XXXXXXXXXXXXXXX., laboratory report no. XXXXXXXXX, XX Xxxxxxxx XXXX (unpublished). Section no. : A 7.1.1.2.1-01	
11.2	**Data protection**	Yes.	
11.2.1	Data owner	LiphaTech S.A.S.	
11.2.2	Companies with letter of access	None.	
11.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		12 GUIDELINES AND QUALITY ASSURANCE	
12.1	**Guideline study**	Yes. The study was performed to ISO 14593.	
12.2	**GLP**	Yes.	
12.3	**Deviations**	No. Apart from minor deviations, the study also meets the requirements of the recommended guidelines (EC methods C.4 A to F or the corresponding OECD 301 A to F guidelines).	
		13 MATERIALS AND METHODS	
13.1	**Test material**	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one.	
13.1.1	Lot/Batch number	DIFM017.	
13.1.2	Specification	No further details.	
13.1.3	Purity	XX.XX% of major distereoisomer.	
13.1.4	Further relevant properties	Not applicable.	
13.2	**Reference substance**	Aniline (Aldrich, batch 68H0112).	
13.2.1	Initial concentration of reference substance	*ca* 25.8 mg/L.	
13.3	**Testing procedure**	The ready biodegradability of difethialone over a period of 28 days was investigated under aerobic conditions at a mean temperature of 20°C.	
13.3.1	Inoculum / test species	The test medium consisted of deionised water with added nutrients as specified in Table A 7.1.1.2.1-1. The inoculum used was secondary effluent sourced from a treatment	

Section A 7.1.1.2.1 Annex Point IIA VII.7.6.1.1	Biodegradability (ready)	
	works which treats sewage of predominantly domestic origin. Effluent was collected overnight on the day before the start of the test. The effluent was filtered to remove solids and sparged with nitrogen for 2 hours whilst maintaining the pH within the range 6.3 to 6.7 (using mineral acid or alkali). Prior to use the pH of the effluent was raised to 7.0 and allowed to settle for at least 1 hour. The inoculum was added to the test medium at 100 mL per litre of final medium.	
13.3.2 Test conditions	For the controls, the inoculated medium (95 mL) was made up to 100 mL with deionised water (5 mL). For the controls with added reference material, a stock solution of aniline (5 mL) was added to the inoculated medium (95 mL). For the treated samples, difethialone (ca 2.9 mg) was added to the inoculated medium (95 mL) which was made up to 100 mL with deionised water (5 mL). The treated test solutions were sealed with butyl rubber septa and placed in an orbital incubator at a nominal temperature of 20°C for 28 days in the dark. At sampling intervals of 4, 7, 14, 21 and 28 days, triplicate test vessels from each treatment group (controls, reference and treated) were taken for analysis. The carbon dioxide present in each sample was quantified by analysis of the liquid phase only, following addition of 10 M sodium hydroxide (1 mL) and shaking for 1 hour. The gaseous inorganic carbon in the headspace of the test vessels is adsorbed into the liquid phase, which is subsequently analysed for total inorganic carbon (TIC). The instrument used was a Dohrman DC 190 analyser.	**x**
	14 RESULTS	
14.1 Degradation of test substance	The extent of biodegradation observed, as measured by the total inorganic carbon content (TIC), is summarised in Table A 7.1.1.2.1-2. The reference substance met the criteria for a readily biodegradable substance during the 28 day incubation period, thereby confirming the validity of the test. The extent of biodegradation of difethialone was < 6% after 28 days. The pass conditions of the test are met if the test substance is biodegraded to the extent > 60% within 10 days in the degradation phase of the test period (the degradation phase follows the lag phase, which is the period from the initiation of the test to attainment of 10% biodegradation). As the biodegradation did not exceed this threshold, difethialone can not be classified as readily biodegradable. As the test was not conducted with both test substance and reference material together, it can not be determined whether difethialone would inhibit the degradation properties of the inoculated medium.	**x** **x**
	15 APPLICANT'S SUMMARY AND CONCLUSION	
15.1 Materials and methods	The ready biodegradability of difethialone over a period of 28 days was investigated under aerobic conditions at a mean temperature of 20°C. The GLP study was conducted to ISO 14593 (equivalent to EC method C.4 C and OECD 301 B, CO_2 headspace test) in 2003.	
15.2 Results and discussion	After 28 days, degradation account for < 6% in a solution containing 20 mg/L difethialone.	**x**

Section A 7.1.1.2.1 **Annex Point IIA VII.7.6.1.1**	**Biodegradability (ready)**	
	The results indicated that difethialone can not be classified as readily biodegradable under the conditions of the test.	
15.3 Conclusion	Difethialone can not be classified as readily biodegradable.	
15.3.1 Reliability	1.	
15.3.2 Deficiencies	None.	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	27 December 2004; revised 31 October 2006	
Materials and Methods	Agree with applicant's summary and conclusion **Comment (3.3.2):** The test concentration was 29 mg/l which is >>0.4 mg/l (solubility of difethialone).	
Results and discussion	Agree with applicant's summary and conclusion **Comment (4.1):** Biodegradation is given as < 6%. It should be specified that this is below the detection level of the method. Significant biodegradation cannot be determined below 10 %. The test was not conducted with a toxicity control and it can not directly be determined whether difethialone in this test would have inhibited the degradation properties of the inoculated medium. However, a respiration inhibition test with activated sludge was conducted (Doc III-A 7.4.1.4-01), showing no effect up to 100 mg/l. It can therefore be concluded that difethialone would not have inhibited biodegradation at the concentration tested (29 mg/l).	
Conclusion	The nominal concentration of difethialone was 29 mg/l, which is far above the water solubility of difethialone. However, no biodegradation was observed. Assuming a maximum dissolved concentration of difethialone at its water solubility it can be that stated that no biodegradation occurred. The high nominal concentration of difethialone is not supposed to have influenced the outcome of the test. Agree with applicant's version	
Reliability	Due to the restrictions noted above the reliability should be changed from 1 to 2.	
Acceptability	Acceptable with the restrictions noted above	
Remarks		

Table A 7.1.1.2.1-1: Composition of test medium

Nutrients	Amount of nutrient per Litre deionised water (mg)
KH_2PO_4	85
K_2HPO_4	217.5
$Na_2HPO_4.2H_2O$	334
NH_4Cl	5
$MgSO_4.7H_2O$	22.5
$CaCl_2.2H_2O$	36.4
$FeCl_3.6H_2O$	0.25
EDTA (disodium salt)	0.40

Table A 7.1.1.2.1-2: Extent of biodegradation observed as measured by total inorganic carbon content (TIC)

Sampling interval (days)	Reference material (20 mg C/L)	Difethialone (20 mg C/L)
4	< 5	< 5
7	66	< 7
14	76	< 5
21	84	< 5
28	89	< 6

Section A 7.1.2.1.2 Annex Point IIIA XII.2.1		Biological sewage treatment, Anaerobic biodegradation	
		16 REFERENCE	**Official use only**
16.1	**Reference**	Xxxxxx, X. and Xxxxxxxxx,XX., XXXXx, Difethialone: Determination of anaerobic biodegradability, Xxxxxxxxxxxxxxxxxx., laboratory report no. XXXXXXXXX, XX Xxxxxx XXXX(unpublished). Section no. : A 7.1.2.1.2-01	
16.2	**Data protection**	Yes.	
16.2.1	Data owner	LiphaTech S.A.S.	
16.2.2	Companies with letter of access	None.	
16.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		17 GUIDELINES AND QUALITY ASSURANCE	
17.1	**Guideline study**	Yes. The study was performed to ISO 11734.	
17.2	**GLP**	Yes.	
17.3	**Deviations**	No. The study was conducted to the recommended guideline (ISO 11734).	
		18 MATERIALS AND METHODS	
18.1	**Test material**	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one..	
18.1.1	Lot/Batch number	Batch DIFM017.	
18.1.2	Specification	No further details.	
18.1.3	Purity	XX.XX% of major distereoisomer.	
18.1.4	Further relevant properties	Not applicable.	
18.2	**Reference substance**	Poly(ethylene glycol), PEG 400. Supplied by Acros Organics (batch no. not stated).	
18.3	**Testing procedure**	The extent of anaerobic biodegradation was investigated in a solution containing difethialone (ca 145 mg/L) incubated at a temperature of 35°C.	**x**
18.3.1	Inoculum / test species	Primary anaerobic digester sludge was obtained from Exeter Sewage Treatment Works (Devon, UK). The sludge was stored at a temperature of 35°C for approximately 7 days prior to use on the study. The day before use on the study the sludge was filtered (1 mm) to remove coarse solids. The filtered diluted sludge contained 13.0 g solids/L (i.e. 1.3% v/v). The filtered sludge was cleaned prior to use by centrifugation (3000 rpm, 5 mins) and reconstitution of the pellet in dilution medium (as prepared in Table A 7.1.2.1.2-1). This procedure was carried out	

Section A 7.1.2.1.2 Annex Point IIIA XII.2.1	Biological sewage treatment, Anaerobic biodegradation	
	twice. The solids were finally suspended in deoxygenated dilution medium (3.5 L) to give a final solids content of 1.9 g/L.	
18.3.2 Test system	Control, reference and test samples were prepared in serum bottles (160 mL). Control samples were prepared by addition of diluted sludge (100 mL). Reference samples were prepared by adding an aliquot (2.0 mL) of a PEG 400 stock solution (9.6 g/L in deionised water) to diluted sludge (100 mL). Test samples were prepared by addition of difethialone (*ca* 14.5 mg) to diluted sludge (100 mL). The diluted sludge contained a redox indicator which was used to identify any oxygen leakage into the sample bottles. Control, reference and test samples were flushed with oxygen free nitrogen to facilitate maintenance of anaerobic conditions. Following equilibration (1 hour) sample bottles were shaken and the internal pressure equalised to atmospheric. Prior to incubation, the diluted sludge inoculum was analysed for inorganic carbon (IC) content and the PEG 400 stock solution was analysed for total organic carbon (TC) content using a Dohrman DC190 carbon analyser.	**x**
18.3.3 Test conditions	Control, reference and test samples were incubated at a temperature of 35°C in the dark in serum bottles. Bottles were shaken each working day. Temperatures readings were taken throughout.	
18.3.4 Sampling	Pressure readings were taken at intervals of 0, 1, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days. Readings were taken using a hand held precision meter (Type 300, Watson & Smith Ltd.). At the end of the incubation period the inorganic carbon in solution was determined using the method previously described.	
18.3.5 Initial TS concentration	Concentration of difethialone in test samples *ca* 145 mg/L (water solubility < 4.8 mg/L, see cross reference Section A 3.5) equivalent to a total carbon content of 100 mg C/L	**x**
	19 RESULTS	
19.1 Degradation of test substance	The incubation temperature was maintained within the range 35 ± 1°C. The extent of biodegradation of difethialone observed, as measured by the amount of organic carbon detected in the headspace and in solution, is summarised in Table A 7.1.2.1.2-2. The reference substance was biodegraded to the extent 83%. The extent of biodegradation of difethialone was 4%, indicating that under the anaerobic conditions of the test, difethialone would not be expected to be degraded by sewage treatment. As the test was not conducted with both test and reference substance together, it can not be determined whether difethialone, at the concentrations used in the test, would inhibit the degradation properties of the sewage sludge.	
	20 APPLICANT'S SUMMARY AND CONCLUSION	
20.1 Materials and methods	Degradation of difethialone by sewage treatment under anaerobic conditions was investigated over a period of 63 days at a mean temperature of 35°C. The GLP study was conducted to ISO 11734 (recommended guideline) in 2003.	

Section A 7.1.2.1.2 **Annex Point IIIA XII.2.1**	**Biological sewage treatment, Anaerobic biodegradation**	
20.2 Results and discussion	After 63 days, degradation account for < 5% in a solution containing 145 mg/L difethialone. The results indicated that difethialone would not be significantly degraded by sewage treatment under anaerobic conditions.	**x**
20.3 Conclusion	Difethialone would not be expected to be significantly degraded by sewage treatment under anaerobic conditions.	**x**
20.3.1 Reliability	1.	
20.3.2 Deficiencies	None.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	3 January 2005; revised 31 October 2006	
Materials and Methods	Agree with applicant's summary and conclusion **Comment (3.3, 3.3.2 and 3.3.5)** The test concentration in this study was 145 mg/l, which is much greater than the water solubility of difethialone, which is 0.39 mg/l. However, it can be assumed that difethialone was dissolved in the test media up to its water solubility and that this part was available for biodegradation. No toxicity control was performed and hence it is unknown whether difethialone inhibits anaerobic degradation at the test concentration in this test. A respiration inhibition test with activated sludge was conducted under aerobic conditions (Doc III-A 7.4.1.4-01), showing no effect up to 100 mg/l. It can be assumed that also under anaerobic conditions no inhibitory effects to primary anaerobic digester sludge occur.	
Results and discussion	Agree with applicant's version	
Conclusion	The nominal concentration of difethialone was 145 mg/l, which is far above the water solubility of difethialone. However, no biodegradation was observed and it can be assumed that the high nominal concentration of difethialone did not influence the outcome of the test. No significant degradation was reported under anaerobic conditions at a concentration of 145 mg/l after 63 days. Agree with applicant's version	
Reliability	Due to the restrictions noted above the reliability should be changed from 1 to 2.	
Acceptability	Acceptable with the restrictions noted above	
Remarks		

Table A 7.1.2.1.2-1: Composition of test dilution medium

Nutrients	Amount of nutrient per Litre deionised water (g)
KH_2PO_4	0.27
$Na_2HPO_4.2H_2O$	0.56
NH_4Cl	0.53
$CaCl_2.2H_2O$	0.075
$MgCl_2.6H_2O$	0.1
$FeCl_2.4H_2O$	0.02
$Na_2S.9H_20$	0.2
Trace nutrient solution[1]	10 mL
(resazurin, redox indicator [2])	0.001

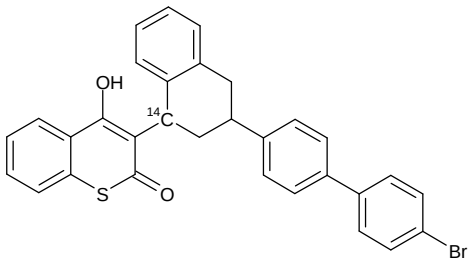
[1] Trace nutrient solution contained $MnCl_2.4H_20$ (0.05 g), H_3BO_3 (0.005 g), $ZnCl_2$ (0.005 g), $CuCl_2$ (0.003 g), $Na_2MoO_4.2H_2O$ (0.001 g), $CoCl_2.6H_2O$ (0.1 g), $NiCl_2.6H_2O$ (0.01 g), Na_2SeO_3 (0.005 g).

[2] Used to identify any oxygen leakage into the sample bottles.

Table A 7.1.1.2.1-2: Extent of biodegradation observed as measured by total inorganic carbon content (TIC)

Sample	pH (mean)	Measured parameters		Calculated parameters (mg)			% biodegradation
		dP (bar)	DIC (mg/L)	C_H	C_L	C_T	
Control	6.67 (6.51 – 6.73)	0.162	34.9	4.6	3.5	8.1	--
Reference	6.50 (6.49 – 6.51)	0.402	49.1	11.5	4.9	16.4	83
Difethialone	6.71 (6.68 – 6.73)	0.172	35.5	4.9	3.6	8.5	4

dP – Difference between initial and final pressure readings.
DIC – Dissolved inorganic carbon in the liquid phase.
C_L – Inorganic carbon in the liquid phase (ignoring the relatively small amount of methane in solution).
C_H – Carbon in the headspace
C_T – Total gasified carbon ($C_L + C_H$).

Section A 7.2.2.1 Annex Point IIIA VII.4, XII.1.1, XII.1.4	Route and rate of degradation in three soils under appropriate conditions	
	21 REFERENCE	**Official use only**
21.1 Reference	Xxxxx, X. and Xxxxxxx, X., XXXX, 14C-Difethialone (LM-2219): Degradation and metabolism in soils incubated under aerobic conditions, Xxxxxxxxxxxxxxxxxx, laboratory report no. XXXXXX, XX Xxxxxxxxx XXXX (unpublished). Section no. : A 7.2.2.1-01	
21.2 Data protection	Yes.	
21.2.1 Data owner	LiphaTech S.A.S.	
21.2.2 Companies with letter of access	None.	
21.2.3 Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
	22 GUIDELINES AND QUALITY ASSURANCE	
22.1 Guideline study	Yes. The study was performed to BBA guideline Part IV, 4-1 (1986).	
22.2 GLP	Yes.	
22.3 Deviations	No. Apart from minor deviations, the study meets the requirements of the recommended guideline (recommended guidelines OCED - draft guideline for aerobic degradation, BBA or US EPA).	
	23 MATERIALS AND METHODS	
23.1 Test material (radiolabelled)	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one. Referred to as LM2219 in the study report.	
23.1.1 Lot/Batch number	Code no. XXX XXXX, batch no. XXXXXXXX.	
23.1.2 Specification	Specific activity 23 mCi/mmol (42.63 mCi/g).	
23.1.3 Purity	RCP (radiochemical purity) XX.X%.	
23.1.4 Further relevant properties	Position of radiolabel given below: 	
23.1.5 Method of analysis	RCP determined prior to use by TLC analysis conditions specified in Section 23.3.4.	

Section A 7.2.2.1 Annex Point IIIA VII.4, XII.1.1, XII.1.4		Route and rate of degradation in three soils under appropriate conditions	
23.2	**Test material (non radiolabelled)**	As given in section 2. Difethialone (IUPAC): 3-((1RS,3RS;1RS,3SR)-3-(4'-bromobiphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxy-1-benzothin-2-one. Referred to as LM2219 in the study report.	
23.2.1	Lot/Batch number	Lot no. XXXXXXXX.	
23.2.2	Specification	No further details.	
23.2.3	Purity	XX.X%.	
23.2.4	Further relevant properties	Not applicable.	
23.3	**Test performance**	The route and rate of aerobic degradation of ^{14}C-difethialone was investigated in two soil types (loamy sand, sand) in the dark under laboratory conditions at a temperature of 20°C and moisture content of 40% maximum water holding capacity (MWHC).	
23.3.1	Test soils	The test soils consisted of Speyer 2.2 soil (loamy sand) and Collombey II soil (sand) types. The characterisation details of the soils are given in Table A 7.2.2.1-1. Prior to use the soils were stored outside. No pesticides had been used on the soils.	
23.3.2	Treatment to soil samples	Soil samples (50 g dry weight), in petri-dishes, were treated with ^{14}C-difethialone (0.198 mg) dissolved in acetone (680 µL). The treatment rate (3.96 mg/kg) is equivalent to an application rate of 2970 g a.s./ha (assuming a soil density of 1.5 g/cm^3 and a mixing depth of 5 cm). The purity of the test material was confirmed before and after treatment. Application was made using a syringe dropwise to the surface of the soil. After application the soil was thoroughly mixed to ensure homogeneity.	
23.3.3	Incubation of soil samples	Following treatment, the moisture content of the soil samples was adjusted to 40% (MWHC). Soil samples were incubated in the dark in a common chamber at a temperature of 20°C. The moisture content of the soils was maintained weekly for the first two weeks and every two weeks subsequently. The microbial biomass of the soils was determined using the respiratory method of Anderson and Domsch (1977)[1] at the start of the study and at the end of the incubation period. Single soil samples were taken for analysis at intervals over a period of 378 days for the Speyer 2.2 soil and 208 days for the Collombey II soil. Soil samples were extracted with acetonitrile (x 2), methanol, methanol: water (80:20 v/v, x 2) and additionally by soxhlet extraction with methanol overnight (if extracted amount was < 90% AR). Extracts were concentrated prior to analysis using two TLC systems. The radioactivity content of the soil extracts and non-extracted soil residue (NER) were quantified using liquid scintillation counting (LSC), and combustion analysis, respectively.	
23.3.4	Chromatographic analysis	Chromatographic analysis of the concentrated soil extracts was conducted by TLC using silica plates (0.25 mm) developed in a number of solvent systems. For routine analysis the following systems were	

[1] Anderson, J.P.E. and Domsch, K.H. (1977). A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biol. Biochem., 10: 215-221.

Section A 7.2.2.1 Annex Point IIIA VII.4, XII.1.1, XII.1.4	Route and rate of degradation in three soils under appropriate conditions	
	used: dichloromethane: chloroform: acetone (30:30:40 v/v/v), n-hexane: diethyl ether: acetic acid (33:65:2 v/v/v), n-hexane: chloroform: acetone (65: 25:10 v/v/v). Non radiolabelled reference standards were visualised using UV light (254 nm). Radioactive regions were quantified using a linear analyser. The RCP determinations were similarly conducted using hexane/ ether/ acetic acid (25/70/5 v/v/v) and methanol/ acetic acid (90/10 v/v).	
	24 RESULTS	
24.1 Recovery and distribution	The recovery and distribution of radioactivity from the soil is summarised in Table A 7.2.2.1-2. The total recovery from soil ranged from 97% to 102% AR (average 99.7%) indicating a complete mass balance. The majority of the radioactivity was extracted from the soil (i.e. >83% AR) at all sampling intervals (i.e. up to 378 days). The level of soil NER was not significant and slowly increased to between 7.4% and 10.9% AR after 378 and 208 days respectively. The lower level of soil NER in this study, as compared to the study described under Section No. A 7.2.1-01, was probably due to the more extensive and complete extraction conditions used in this case. The maximum level of soil NER reached only 10.9% AR after 208 days, therefore, no further bound residue fractionation was conducted. The level of volatile components evolved was minimal at all sampling intervals, no organic volatiles were detected and the level of carbon dioxide evolved comprised up to 2.8% AR after 208 days. The levels of soil NER and evolved volatile components are therefore not considered significant.	**x**
24.2 Profile of components	The profile of components extracted from the soil samples, as determined by TLC, is summarised in Table A 7.2.2.1-3. The levels of difethialone observed in the soils declined slowly and comprised 55% AR in the Speyer 2.2 soil and 41.6% AR in the Collombey II soil after 378 and 208 days, respectively. The levels of difethialone observed were confirmed using HPLC analysis, see Table A 7.2.2.1-4. A reasonably good agreement was obtained, confirming the approximate amounts of difethialone detected in the soils.	
24.3 Route of degradation	Full characterisation of the extracted components was only conducted in the Speyer 2.2 soil. Degradation of difethialone led to the formation of two significant metabolites, M3 and M4 which were not identified further. Levels of the component M3 plateaued between 8% and 14% AR over the period 14 to 189 days. Levels of the component M4 increased to a maximum of 10% AR after 140 days and subsequently declined to 5% after 378 days. At least five minor components were also observed at individual maximum levels ranging between 4% and 9.6% AR.	
24.3.1 Significant degradation products	Two metabolites (M3 and M4) were observed in significant quantities (i.e. ≥ 10% AR).	x

Section A 7.2.2.1 Annex Point IIIA VII.4, XII.1.1, XII.1.4	Route and rate of degradation in three soils under appropriate conditions	
24.4 Rate of degradation	The rate of degradation of difethialone was recalculated using the procedure described in Section A 7.2.1-01. The recalculation of the DT_{50} and DT_{90} values is presented graphically in Figures A 7.2.2.1-1 and A 7.2.2.1-2 and summarised in Table A 7.2.2.1-5. The degradation of difethialone, at a temperature of 20°C and moisture content of 40% MWHC, did not give a good correlation to first-order kinetics ($R^2 \leq 0.54$). The best fit first order half life of difethialone in soil was 224 and 524 days for the Speyer 2.2 and Collombey II soils, respectively. To reflect an average EU outdoor temperature of 12°C, the degradation rates have been converted using the Arrhenius equation with a default activation energy of 54.0 kJ/mol. Converted to a temperature of 12°C the DT_{50} and DT_{90} values for the Speyer 2.2 / Collombey II soils were 976 / 417 days and 3244 / 1390 days, respectively.	
	25 APPLICANT'S SUMMARY AND CONCLUSION	
25.1 Materials and methods	The route and rate of aerobic degradation of ^{14}C-difethialone was investigated in two soil types (loamy sand, sand) in the dark under laboratory conditions at a temperature of 20°C and moisture content of 40% MWHC. The GLP study was conducted to the BBA Guideline Part IV, 4-1 in 1993.	
25.2 Results and discussion	The total recovery from soil ranged from 97% to 102% AR (average 99.7%) indicating a complete mass balance. The levels of difethialone observed declined slowly with DT_{50} values equivalent to 976 and 417 days in the Speyer 2.2 and Collombey II soils, respectively, at a temperature of 12°C. Degradation of difethialone produced two significant metabolites M3 and M4 which were observed at maximum levels of 13.5% and 10.2% AR after 189 and 140 days, respectively. These metabolites were not identified further. Degradation of difethialone led to the formation of minor amounts of soil NER and evolved volatile components.	x
25.3 Conclusion	Difethialone is slowly degraded in soil under aerobic conditions. Two metabolites were formed in quantities greater than 10% AR, however these metabolites were not identified further. Up to 5 other minor components were also observed at levels not exceeding 10% AR. Degradation of difethialone led to the formation of minor amounts of soil NER and evolved volatile components.	x
25.3.1 Reliability	2.	
25.3.2 Deficiencies	Yes. The study contained some deficiencies when compared to modern day standards. These deficiencies are discussed in more detail where appropriate under the relevant headings above and are not considered to have adversely affected the quality of the results.	

Section A 7.2.2.1 **Annex Point IIIA VII.4, XII.1.1, XII.1.4**	**Route and rate of degradation in three soils under appropriate conditions**	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	21 December 2004; revised 31 October 2006	
Materials and Methods	Agree with applicant's version	
Results and discussion	Agree applicant's summary and conclusion. **Comment (4.1 and 5.2):** NER was >10 % at the final sample point (10.9 % at day 208), in one soil. NER was increasing in time and still may increase after the end of the study. According to data requirement for biocidal products, chapter 3, section 7.2.2.3 this should trigger further studies into the nature of the bound residues. **Comment (4.3.1):** Degradation of difethialone led to the formation of two unidentified metabolites which were present in significant quantities (i.e. exceeding 10 % applied radioactivity) for up to 189 days at a temperature of 20°C.	
Conclusion	**Comment (5.3):** Difethialone is slowly degraded in soil under aerobic conditions. Two metabolites were formed in quantities greater than 10% AR, however, these metabolites were not identified further. Up to 5 other minor components were also observed at levels not exceeding 10% AR. Degradation of difethialone led to the formation of NER >10 % only at the final sampling point (10.9 % at day 208). As the risk associated with possible metabolites or bound residues is assumed to be less than for the active compound (the highly specific mode of action of difethialone, is probably destroyed/reduced in any metabolite) and the exposure to soil is restricted to only small areas, no further studies are needed to investigate metabolites and bound residues. The main route of degradation was not identified. However, as the risk associated with metabolites is assumed to be less than for the active compound no further testing is needed.	
Reliability	2	
Acceptability	Acceptable with the restrictions noted above	
Remarks		

Table A 7.2.2.1-1: Classification and physico-chemical properties of soils used as adsorbents

Soil name	**Speyer 2.2**	**Collombey II**
Source	Germany	Switzerland
Textural classification, USDA	loamy sand	sand
Sand [%]	78.8	86.0
Silt [%]	12.4	12.7
Clay [%]	8.8	1.3
Textural classification, ISSS	n.d	n.d
Sand [%]	88.0	94.3
Silt [%]	7.1	4.4
Clay [%]	4.9	1.3
Organic carbon [%]	2.55	1.33
pH	6.0	7.7
Cation exchange capacity (MEQ/100 g)	7.2	11.2
Moisture content, (g/100 g soil) Max. water holding capacity (MWHC). 40% MWHC	 40.4 16.2	 42.0 16.8
Microbial biomass (mg C/100 g soil) pre-study after *ca* 130 days end of study	 36.9 13.3 10.9	 60.6 -- 15.1

n.d – not determined.

Table A 7.2.2.1-2: Recovery and distribution of radioactivity from aerobic soil samples

Sample times (days)	Extractable (% AR)				Soil NER (% AR)	Volatile components		Total (% AR)[1]
	Organic	Aqueous	Soxhlet	Sub-total		Organic volatiles	CO_2	
Speyer 2.2 – loamy sand								
0	n.p	93.1	n.p	(93.1)	5.3	n.d	n.d	98.4
14	85.2	4.6	5.8	(95.6)	2.7	< 0.1	0.8	99.1
28	83.9	4.8	6.8	(95.4)	3.3	< 0.1	1.0	99.7
56	84.1	4.5	8.3	(97.0)	3.7	< 0.1	1.1	101.8
98	81.3	4.3	9.2	(94.8)	4.2	< 0.1	1.5	100.5
140	80.8	4.7	8.0	(93.4)	4.8	< 0.1	1.7	99.9
189	80.5	4.0	8.0	(92.5)	4.6	< 0.1	1.9	98.9
378	77.6	5.2	8.2	(91.0)	7.4	< 0.1	2.5	100.8
Collombey II – sand								
0	n.p	96.8	n.p	(96.8)	2.6	n.d	n.d	99.5
7	83.5	7.2	6.4	(97.1)	3.0	< 0.1	0.4	100.4
14	80.9	8.2	6.8	(95.8)	3.3	< 0.1	0.6	99.7
28	78.3	8.2	7.8	(94.4)	5.0	< 0.1	0.7	100.1
49	75.8	8.5	8.9	(93.2)	6.5	< 0.1	1.0	100.7
77	73.4	7.7	9.9	(90.9)	6.9	< 0.1	1.4	99.2
105	69.2	9.4	9.6	(88.1)	9.3	< 0.1	1.8	99.3
208	65.1	7.9	10.1	(83.1)	10.9	< 0.1	2.8	96.7

Values are from single samples at each sampling interval.

n.d – not determined.

n.p – not performed.

[1] The overall range of the total recovery from the individual samples was 96.7% to 101.8% (overall average 99.7%).

Table A 7.2.2.1-3: Profile of radioactivity extracted from aerobic soil samples

Sample times (days)	Soil components (% AR)								Total[1] (% AR)
	Parent	M1	M2	M3	M4	M5	M6	M7	
Speyer 2.2 – loamy sand									
0	89.7	n.d	n.d	n.d	n.d	n.d	n.d	3.4	93.1
14	69.8	n.d	4.2	7.6	6.3	n.d	0.2	7.6	95.6
28	65.5	n.d	6.0	12.1	5.6	n.d	0.4	5.8	95.4
56	68.9	3.1	6.7	8.0	6.2	n.d	n.d	4.0	97.0
98	66.9	2.7	4.4	8.3	6.5	0.3	0.4	5.2	94.8
140	55.9	1.7	5.9	8.0	10.2	4.0	n.d	7.9	93.6
189	48.4	2.6	7.0	13.5	4.4	3.1	4.0	9.6	92.5
378	55.0	5.9	8.3	3.9	4.9	n.d	5.3	7.7	91.0

Sample times (days)	Soil components (% AR)			Total[1] (% AR)
	Parent	Polar residues	Non-polar residues	
Collombey II – sand				
0	92.2	4.6	n.d	96.8
7	72.3	5.4	19.5	97.1
14	47.9	16.1	31.8	95.8
28	60.4	17.1	16.9	94.4
49	54.4	10.0	28.8	93.2
77	53.6	14.6	22.8	90.9
105	54.1	13.6	20.5	88.1
208	41.6	11.9	29.6	83.1

n.d – not determined.

Table A 7.2.2.1-4: Confirmatory analysis of Speyer 2.2 soil by HPLC

Sample times (days)	Difethialone (% AR)
0	69.3
14	65.7
28	64.4
56	64.2
98	60.9
140	49.3
189	50.0
378	49.8

Figure A 7.2.2.1-1: Re-calculation of DT_{50} and DT_{90} values for Speyer 2.2 soil using first-order kinetics

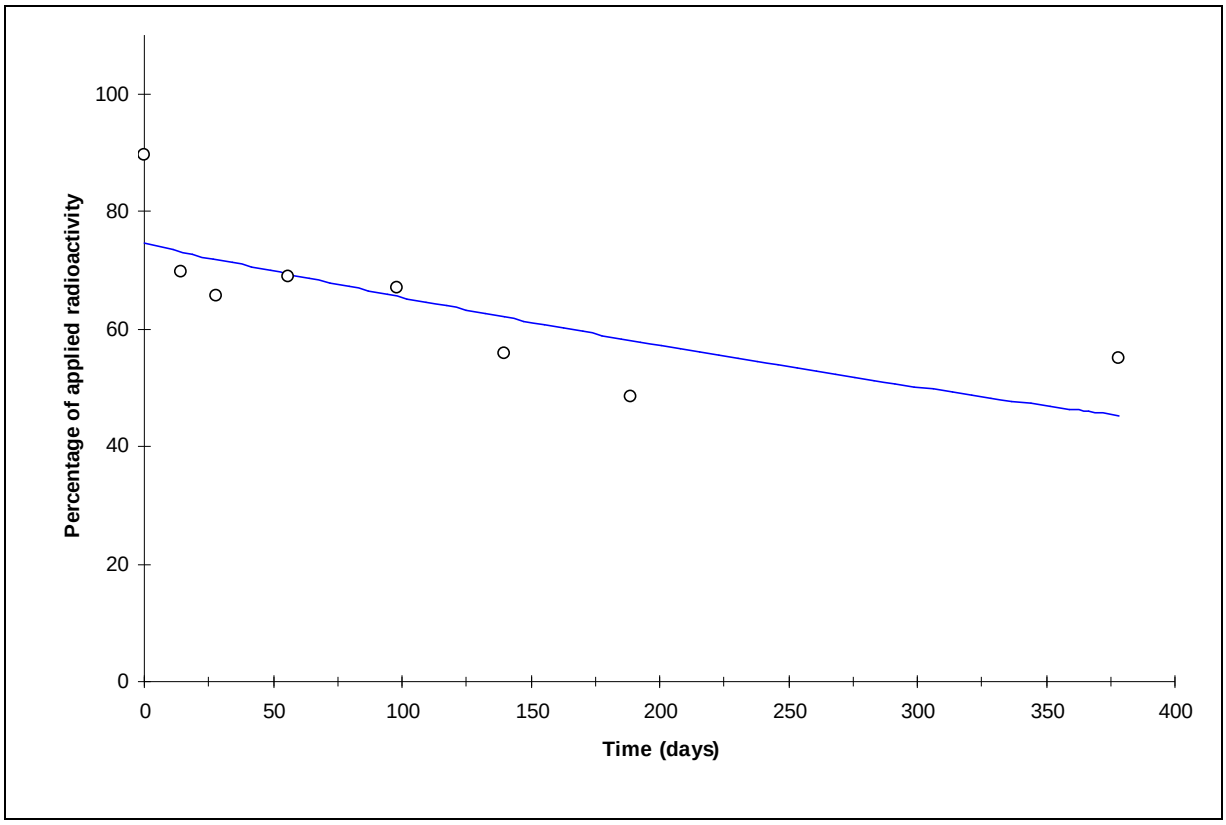


Figure A 7.2.2.1-2: Re-calculation of DT_{50} and DT_{90} values for Collombey II soil using first-order kinetics

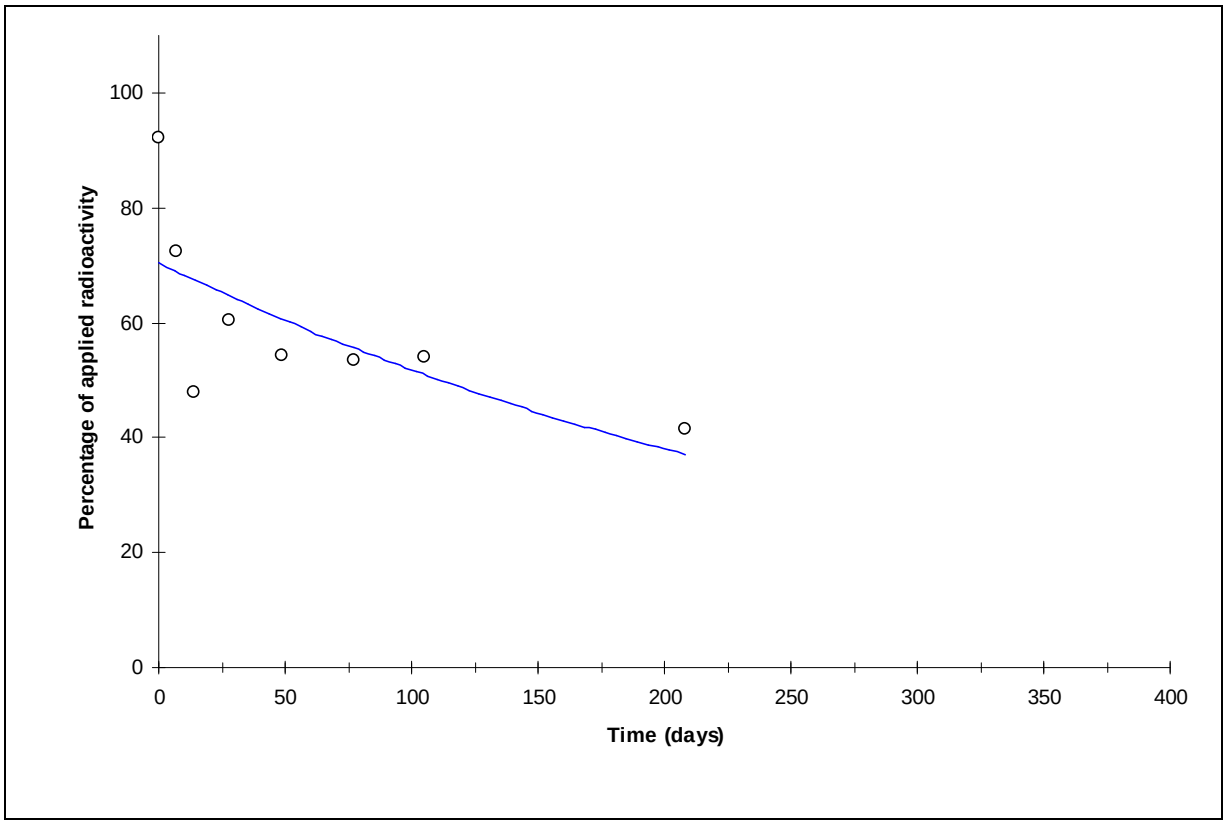


Table A 7.2.2.1-5: First order $DT_{50(lab)}$ and $DT_{90(lab)}$ values for the rate of aerobic degradation of difethialone in soil

Soil type	Data range (days)	$DT_{50(lab)}$ (days)	$DT_{90(lab)}$ (days)	Regression parameters		
				C_0	k	R^2
Speyer 2.2 (loamy sand)	0 to 378	524[1]	1741[1]	74.56	0.00132	0.54
Collombey II (sand)	0 to 208	224[1]	746[1]	70.50	0.00309	0.46

The soil was incubated at a temperature of 20°C and a moisture content of 40% MWHC.

[1] DT_{50} (or DT_{90}) value was not demonstrated experimentally, result obtained by extrapolation.

Section A7.4.1.1-01 Annex Point IIA VII.7.1		Acute toxicity to fish	
		26 REFERENCE	**Official use only**
26.1	**Reference**	Xxxxx, XX. and Xxxxxxxx, X. (XXXX). [^{14}C]-difethialone: Determination of acute toxicity (LC_{50}) to rainbow trout (96 h, semi-static) XXXXXXXXXX, laboratory report number XXXXX, XX Xxxxx XXXX (unpublished).	
26.2	**Data protection**	Yes.	
26.2.1	Data owner	LiphaTech S.A.S.	
26.2.2	Companies with letter of access	None.	
26.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		27 GUIDELINES AND QUALITY ASSURANCE	
27.1	**Guideline study**	Yes. OECD 203 and EU C.1.	
27.2	**GLP**	Yes.	
27.3	**Deviations**	None.	
		28 MATERIALS AND METHODS	
28.1	**Test material**	[^{14}C]-difethialone (1.92 GBq/mmol) combined with non-radiolabelled difethialone.	
28.1.1	Lot/Batch number	XXXXXXX ([^{14}C]-difethialone); XXXXXXXXXX (non-radiolabelled difethialone).	
28.1.2	Purity	[^{14}C]-difethialone re-purified to RCP of XX.XX% prior to use; non-radiolabelled difethialone: XX.X% (w/w).	
28.1.3	Further relevant properties	Photolysis half-life of difethialone is less than 1 hour, log octanol:water partition coefficient is 6.29 and water solubility is < 4.8 mg/L at 20°C.	**x**
28.2	**Preparation of TS solution for poorly soluble or volatile test substances**	A range of stock solutions was prepared with 10.26, 22.70, 49.43, 108.25 and 239.61 mg non-radiolabelled difethialone/1.0 mL dimethylformamide. 400 µL of a stock solution containing [^{14}C]-difethialone was added to each and all volumes made to 5.0 mL with additional organic solvent. Dosing stocks were transferred to the medium in the appropriate test tanks at a rate of 1.0 mL/12 L.	
28.3	**Reference substance**	No.	
28.4	**Testing procedure**		
28.4.1	Dilution water	See Table A7.4.1.1-2.	
28.4.2	Test organisms	See Table A7.4.1.1-3.	
28.4.3	Test system	See Table A7.4.1.1-4.	
28.4.4	Test conditions	See Table A7.4.1.1-5.	

Section A7.4.1.1-01 **Annex Point IIA VII.7.1**		**Acute toxicity to fish**	
28.4.5	Duration of the test	48 hours. (100% mortality occurred at all test concentrations within this time).	
28.4.6	Test parameter	Mortality and observations of toxicity.	
28.4.7	Monitoring of TS concentration	By liquid scintillation counting, and with LSC data converted to mg difethialone-equivalents/L. Stability trials were conducted before the study, with [^{14}C]-difethialone in either dimethylformamide or reconstituted test water at concentrations of between 0.17 and 4.0 mg/L and refrigerated in darkness for 5 days prior to analysis by HPLC with UV detection. UV traces obtained for difethialone in both media showed no change in retention time and the absence of any major metabolites. Difethialone was therefore presumed to remain substantially intact under test conditions.	
28.4.8	Statistics	Not reported.	
		29 RESULTS	
29.1	**Results test substance**		
29.1.1	Effect data (Mortality)	See Table A7.4.1.1-7.	
29.1.2	Other effects	See Table A7.4.1.1-7.	
29.2	**Results of controls**		
29.2.1	Number/ percentage of animals showing adverse effects	None.	
29.3	**Test with reference substance**	Not performed.	
		30 APPLICANT'S SUMMARY AND CONCLUSION	
30.1	**Materials and methods**	Semi-static acute toxicity test with rainbow trout in accordance with OECD 203 and EU method C.1.	
30.2	**Results and discussion**		
30.2.1	LC_{50}	48-hour (and 96-hour) LC_{50} < 0.15 mg/L, based on mean measured concentrations.	**x**
30.3	**Conclusion**	See Table A7.4.1.1-9.	
30.3.1	Reliability	1	**x**
30.3.2	Deficiencies	More than 50% mortality occurred within 48 hours at all concentrations tested. Consequently, it was not possible to determine a 96 hour LC_{50} endpoint from the results of this study.	

Section A7.4.1.1-01 **Annex Point IIA VII.7.1**	**Acute toxicity to fish**	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	13 December 2004; revised 31 October 2006	
Materials and Methods	Agree with applicant's version **Comment (3.1.3):** The water solubility of difethialone is set to be 0.39 mg/l.	
Results and discussion	48-hour LC_{50} < 0.15 mg/L, based on mean measured concentrations.	
Conclusion	The present study cannot be used for risk assessment purposes as mortality exceeded 50 % at all tested concentrations and the exposure period was only 48 hours. The test should have been performed at lower concentrations.	
Reliability	Due to the restrictions noted above, the reliability should be changed from 1 to 3. According to TNsG on Dossier Preparation and Study Evaluation, part I, is reliability indicator 3 "a study with major methodological and /or reporting deficiencies". The test has not been performed according to standard guideline which requires that a range finding test should be performed in order to ensure a proper range of test concentrations. Test period is <96 hours.	
Acceptability	Test is not acceptable as endpoint for acute fish toxicity as a LC_{50} (96hour) has not been observed. Other tests are available for this endpoint giving acceptable results.	
Remarks		

Table A7.4.1.1-1: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Vehicle	Dimethylformamide.
Concentration of vehicle	0.08 mL/L
Vehicle control performed	Yes, 0.08 mL dimethylformamide/L

Table A7.4.1.1-2: Dilution water

Criteria	Details
Source	Reconstituted water.
Alkalinity	31 mg $CaCO_3$/L.
Hardness	81 mg $CaCO_3$/L.
Ph	7.69
Conductance	183 µS/cm.
Holding water different from dilution water	No.

Table A7.4.1.1-3: Test organisms

Criteria	Details
Species/strain	*Oncorhynchus mykiss*.
Source	Commercial supplier in UK.
Age/size	Length 40-60 mm.
Pretreatment	Holding period ≥ 12 days
Feeding of animals during test	No. Feeding stopped 24 hours prior to the start of the test.

Table A7.4.1.1-4: Test system

Criteria	Details
Test type	Semi-static.
Volume of test vessels	14 L silanised glass tanks containing 12 L medium.
Volume/animal	1.7 L
Number of animals/vessel	7.
Number of vessels/ concentration	1

Table A7.4.1.1-5: Test conditions

Criteria	Details
Test temperature	13.8 to 14.9°C
Dissolved oxygen	83.8 to 100% ASV.
pH	6.44 to 7.90.
Photoperiod	24 h darkness except at observation and maintenance times.

Table A7.4.1.1-6: Concentrations of [^{14}C]-difethialone measured in fresh and expired (24 hour) media samples

Test Substance Concentration [mg/L]	Measured concentration[a]			Recovery	
	0 hours (fresh)	24 hours (expired)	Mean	Initial as % of nominal	24 hour as % of initial
Control	< lod	< lod	< lod	na	na
Solvent control	< lod	< lod	< lod	na	na
0.17	0.16	0.13	0.15	94	76
0.38	0.38	0.28	0.33	100	74
0.83	0.78	0.66	0.72	94	80
1.8	1.75	1.62	1.69	97	90
4.0	3.96	3.54	3.75	99	89

[a] mg equivalent/L;
< lod: below limit of detection by LSC (30 dpm);
na: not applicable.

Table A7.4.1.1-7: Mortality data

Test Substance Concentration [mg/L]		Mortality (out of 7 fish per treatment)			
Nominal	**Mean measured**	**1 hour**	**4 hours**	**24 hours**	**48 hours[a]**
Control	-	0	0	0	0
Solvent control	-	0	0	0	0
0.17	0.15	0	0	0	5
0.38	0.33	0	0	2[b]	7
0.83	0.72	0	0	7	7
1.8	1.68	0	0	7	7
4.0	3.75	0	5	7	7

[a] Mortality at all difethialone concentrations exceeded 50% and study was therefore terminated prior to 96 h.
[b] Survivors displayed loss of equilibrium.

Table A7.4.1.1-8: Effect data

Parameter	**24 h**	**48 h**
LC_{50} [mg/L][1]	0.35	< 0.15
95 % c.l.	0.31 to 0.40	n.a.

[1] Based on mean measured concentrations.

Table A7.4.1.1-9: Validity criteria for acute fish test according to OECD Guideline 203

	fulfilled	**Not fulfilled**
Mortality of control animals <10%	**Yes**	
Concentration of dissolved oxygen in all test vessels > 60% saturation	**Yes**	
Concentration of test substance ≥80% of initial concentration during test		**No (74 – 93%)**

Section A7.4.1.1-02 Annex Point IIA VII.7.1		Acute toxicity to fish	
		31 REFERENCE	**Official use only**
31.1	**Reference**	Xxxxxxxxxx, XX. and Xxxxxxxxx, X (XXXXx). Acute toxicity of LM-2219 to rainbow trout (*Salmo gairdneri*). XXXXXXXXXXXXXXXXXXXXXXXXXXX laboratory report number XXXXXXXXXXXXXXXXXXXX, XX Xxxxxxx XXXX (unpublished).	
31.2	**Data protection**	Yes.	
31.2.1	Data owner	LiphaTech S.A.S.	
31.2.2	Companies with letter of access	None.	
31.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		32 GUIDELINES AND QUALITY ASSURANCE	
32.1	**Guideline study**	Yes. ASTM (1980), comparable to OECD 203 and EU C.1.	
32.2	**GLP**	Yes.	
32.3	**Deviations**	No analysis of test media for difethialone and concentration of dissolved oxygen fell below 60% ASV.	
		33 MATERIALS AND METHODS	
33.1	**Test material**	Difethialone (termed LM-2219 in the report).	
33.1.1	Lot/Batch number	XXX XXXX.	
33.1.2	Purity	XX.X% (w/w).	
33.1.3	Further relevant properties	Photolysis half-life of difethialone is less than 1 hour, log octanol:water partition coefficient is 6.29 and water solubility is < 4.8 mg/L at 20°C.	**x**
33.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Stock solution prepared with 1,000 µg/mL in dimethylformamide. Stock solution then added to dilution water and stirred.	
33.3	**Reference substance**	No.	
33.4	**Testing procedure**		
33.4.1	Dilution water	See Table A7.4.1.1-11.	
33.4.2	Test organisms	See Table A7.4.1.1-12.	
33.4.3	Test system	See Table A7.4.1.1-13.	
33.4.4	Test conditions	See Table A7.4.1.1-14.	
33.4.5	Duration of the test	96 hours.	
33.4.6	Test parameter	Mortality and observations of toxicity.	

Section A7.4.1.1-02 **Annex Point IIA VII.7.1**		**Acute toxicity to fish**	
33.4.7	Monitoring of TS concentration	No.	
33.4.8	Statistics	LC_{50} by non-linear interpolation (software of Stephan 1982).	
		34 RESULTS	
34.1	**Results test substance**		
34.1.1	Effect data (Mortality)	See Table A7.4.1.1-15.	
34.1.2	Other effects	See Table A7.4.1.1-15.	
34.2	**Results of controls**		
34.2.1	Number/ percentage of animals showing adverse effects	None.	
34.3	**Test with reference substance**	Not performed.	
		35 APPLICANT'S SUMMARY AND CONCLUSION	
35.1	**Materials and methods**	Static acute toxicity test with rainbow trout in accordance with OECD 203 and EU method C.1. No analysis of test media for difethialone and dissolved oxygen concentration fell below 60% ASV.	
35.2	**Results and discussion**		
35.2.1	LC_{50}	96-hour LC_{50} = 51 µg/L (95% confidence limits of 36 to 100 µg/L).	x
35.3	**Conclusion**	See Table A7.4.1.1-17.	
35.3.1	Reliability	2	
35.3.2	Deficiencies	Yes. No measurement of test substance in test medium. Results can be interpreted in relation to study summarised in A.7.4.1.1-01. Fall in dissolved oxygen concentration was only slightly below 60% and not sufficient to alter the outcome of the test.	
		Evaluation by Competent Authorities	
		Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
		EVALUATION BY RAPPORTEUR MEMBER STATE	
Date		13 December 2004; revised 31 October 2006	
Materials and Methods		Agree with applicant's version **Comment (3.1.3):** The water solubility of difethialone is set to be 0.39 mg/l	

Section A7.4.1.1-02 Annex Point IIA VII.7.1	Acute toxicity to fish	
Results and discussion	Agree with applicant's version **Comment (5.2.1):** The study is not fully valid as the test concentrations have not been verified analytically. Moreover, the static test design is not preferable for the testing of difficult substances like difethialone. The real exposure concentrations in test are likely to have slightly decreased over the test period mainly due to the low water solubility of difethialone and possibly due to adsorption of the test substance to glassware. The toxicity of difethialone to fish might therefore be underestimated. In another study, conducted under semi-static conditions (A7.4.1.1-01), 14C-difethialone was used to facilitate the monitoring of exposure concentrations. Recovery rates after 24 hours were between 74 and 90% but no measurements are available for a later point in time during the test.	
Conclusion	Agree with applicant's version	
Reliability	2	
Acceptability	Acceptable with the restrictions noted above	
Remarks	The dissolved oxygen concentration of some of the treatments was measured at slightly below 60% of saturation (57-59%). This is not considered to be sufficient to alter the outcome of the test.	

Table A7.4.1.1-10: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Vehicle	Dimethylformamide.
Concentration of vehicle	0.1 mL/L
Vehicle control performed	Yes, 0.1 mL dimethylformamide/L

Table A7.4.1.1-11: Dilution water

Criteria	Details
Source	Reconstituted soft water (ASTM 1980).
Alkalinity	32 mg $CaCO_3$/L.
Hardness	47 mg $CaCO_3$/L.
pH	7.7
Conductance	160 µmhos/cm.
Holding water different from dilution water	Yes, well water, hardness 32-34 mg $CaCO_3$/L, alkalinity 25-30 mg $CaCO_3$/L, conductivity 110 µmhos/cm, pH 6.8-7.0.

Table A7.4.1.1-12: Test organisms

Criteria	Details
Species/strain	*Oncorhynchus mykiss* (formerly known as *Salmo gairdneri*).
Source	Commercial supplier in CA, USA.
Age/size	Mean wet weight 0.72 g, total length 31-46 mm.
Pretreatment	14 days holding period.
Feeding of animals during test	No.

Table A7.4.1.1-13: Test system

Criteria	Details
Test type	Static.
Volume of test vessels	15 L
Volume/animal	1.5 L
Number of animals/vessel	10
Number of vessels/ concentration	1

Table A7.4.1.1-14: Test conditions

Criteria	Details
Test temperature	13°C.
Dissolved oxygen	57 to 86% ASV, less than 60% ASV in two vessels only at 72 to 96 hours.
pH	6.9 to 7.6
Photoperiod	16 h daily.

Table A7.4.1.1-15: Mortality data

Nominal Test Substance Concentration [µg/L]	Mortality (out of 10 fish per treatment)			
	24 hours	48 hours	72 hours	96 hours
Control	0	0	0	0
Solvent control	0	0	0	0
8.0	0	0	0	0
13	0	0	0	0
22	0	0	0	0
36	0	0	0	0[e]
60	1[a]	3[a,b]	6[a,b,c]	8[c,d]
100	7[a]	10	10	10

[a] All survivors showed loss of equilibrium and excessive mucous production.
[b] All survivors at the surface of the test medium.
[c] One fish lethargic.
[d] One fish with darkened pigmentation and at the surface of the test medium.
[e] Several fish showing darkened pigmentation.

Table A7.4.1.1-16: Effect data

Parameter	96 h [µg/L][1]	95 % c.l.
LC_{50}	51	36 to 100

[1] Based on nominal concentrations.

Table A7.4.1.1-17: Validity criteria for acute fish test according to OECD Guideline 203

	fulfilled	Not fulfilled
Mortality of control animals <10%	**Yes**	
Concentration of dissolved oxygen in all test vessels > 60% saturation	**No**	
Concentration of test substance ≥80% of initial concentration during test	**Not measured**	

Section A7.4.1.1-03 Annex Point IIA VII.7.1		Acute toxicity to fish	
		36 REFERENCE	**Official use only**
36.1	**Reference**	Xxxxxxxxxx, XX. and Xxxxxxxxx, X. (XXXXx). Acute toxicity of LM-2219 to bluegill (*Lepomis macrochirus*). XXXXXXXXXXXXXXXXXXXXXXXXXXXX, laboratory report number XXXXXXXXXXXXXXXXX, XX Xxxxxxxx XXXX (unpublished).	
36.2	**Data protection**	Yes.	
36.2.1	Data owner	LiphaTech S.A.S.	
36.2.2	Companies with letter of access	None.	
36.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		37 GUIDELINES AND QUALITY ASSURANCE	
37.1	**Guideline study**	Yes. ASTM (1980), comparable to OECD 203 and EU C.1.	
37.2	**GLP**	Yes.	
37.3	**Deviations**	Yes. No analysis of test media for difethialone	x
		38 MATERIALS AND METHODS	
38.1	**Test material**	Difethialone (termed LM-2219 in the report).	
38.1.1	Lot/Batch number	XXXXXXXX.	
38.1.2	Purity	XX.X% (w/w).	
38.1.3	Further relevant properties	Photolysis half-life of difethialone is less than 1 hour, log octanol-water partition coefficient is 6.29 and water solubility is 0.39 mg/L at 25°C.	x
38.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Stock solution prepared with 6.0 µg/mL in dimethylformamide. Stock solution then added to dilution water and stirred.	
38.3	**Reference substance**	No.	
38.4	**Testing procedure**		
38.4.1	Dilution water	See Table A7.4.1.1-19.	
38.4.2	Test organisms	See Table A7.4.1.1-20.	
38.4.3	Test system	See Table A7.4.1.1-21.	
38.4.4	Test conditions	See Table A7.4.1.1-22.	
38.4.5	Duration of the test	96 hours.	
38.4.6	Test parameter	Mortality and observations of toxicity.	
38.4.7	Monitoring of TS concentration	No.	
38.4.8	Statistics	LC_{50} by non-linear interpolation (software of Stephan 1982).	

Section A7.4.1.1-03 **Annex Point IIA VII.7.1**		**Acute toxicity to fish**	
		39 RESULTS	
39.1	**Results test substance**		
39.1.1	Effect data (Mortality)	See Table A7.4.1.1-23.	
39.1.2	Other effects	See Table A7.4.1.1-23.	
39.2	**Results of controls**		
39.2.1	Number/ percentage of animals showing adverse effects	None.	
39.3	**Test with reference substance**	Not performed.	
		40 APPLICANT'S SUMMARY AND CONCLUSION	
40.1	**Materials and methods**	Static acute toxicity test with bluegill sunfish in accordance with OECD 203 and EU method C.1. No analysis of test media for difethialone and concentration of dissolved oxygen less than 60% ASV.	
40.2	**Results and discussion**		
40.2.1	LC_{50}	96-hour LC_{50} = 75 µg/L (95% confidence limits of 48 to 130 µg/L).	x
40.3	**Conclusion**	See Table A7.4.1.1-24.	
40.3.1	Reliability	2	
40.3.2	Deficiencies	Yes. No measurement of test substance in test medium. Results can be interpreted in relation to study summarised in A.7.4.1.1-01. Fall in dissolved oxygen concentration over time is not considered to be sufficient to alter the outcome of the toxicity test.	
		Evaluation by Competent Authorities	
		Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
		EVALUATION BY RAPPORTEUR MEMBER STATE	
Date		13 December 2004; 31st October 2006	

Section A7.4.1.1-03 Annex Point IIA VII.7.1	Acute toxicity to fish	
Materials and Methods	Agree with applicant's version **Comment (2.3):** The concentration of dissolved oxygen during the test fell below 60% saturation level (32 - 59%) in two vessels at 72 to 96 hours. However, the toxic effects of difethialone occur already after 24/48 hours and hence the decrease in oxygen content after 72 to 96 hours is not considered to have affected the outcome of the test. **Comment (5.2.1):** The study is not fully valid as the test concentrations have not been verified analytically. Moreover, the static test design is not preferable for the testing of difficult substances like difethialone. The real exposure concentrations in test are likely to have slightly decreased over the test period mainly due to the low water solubility of difethialone and possibly due to adsorption of the test substance to glassware. The toxicity of difethialone to fish might therefore be underestimated.	
Results and discussion	Agree with applicant's version	
Conclusion	Agree with applicant's version	
Reliability	2	
Acceptability	Acceptable	
Remarks	The dissolved oxygen concentration of some of the treatments was measured at below 60% of saturation (32-59%). Although nominal concentrations were not measured the test was performed as GLP study and difethialone has been shown to be stable in aqeous solution within the present timeframe of the test. However it is likely that a somewhat lower LC50 might have been observed in a semistatic test given the high Log Kow of this compound.	

Table A7.4.1.1-18: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Vehicle	Dimethylformamide.
Concentration of vehicle	0.1 mL/L
Vehicle control performed	Yes, 0.1 mL dimethylformamide/L

Table A7.4.1.1-19: Dilution water

Criteria	Details
Source	Reconstituted soft water (ASTM 1980).
Alkalinity	29 mg $CaCO_3$/L
Hardness	49 mg $CaCO_3$/L
pH	7.1
Conductance	220 µmhos/cm
Holding water different from dilution water	Yes, well water, hardness 24-34 mg $CaCO_3$/L, alkalinity 22-23 mg $CaCO_3$/L, conductivity 100-170 µmhos/cm, pH 7.0-7.1.

Table A7.4.1.1-20: Test organisms

Criteria	Details
Species/strain	*Lepomis macrochirus*
Source	Commercial supplier in MO, USA
Age/size	Mean wet weight 0.52 g, total length 27-40 mm
Pretreatment	14 days minimum holding period
Feeding of animals during test	No

Table A7.4.1.1-21: Test system

Criteria	Details
Test type	Static
Volume of test vessels	15 L
Volume/animal	1.5 L
Number of animals/vessel	10
Number of vessels/ concentration	1

Table A7.4.1.1-22: Test conditions

Criteria	Details
Test temperature	22°C
Dissolved oxygen	32 to 98% ASV, less than 60% ASV in two vessels only at 72 to 96 hours
pH	6.8 to 7.8
Photoperiod	16 h daily

Table A7.4.1.1-23: Mortality data

Nominal Test-Substance Concentration [µg/L]	Mortality (out of 10 fish per treatment)			
	24 hours	48 hours	72 hours	96 hours
Control	0	0	0	0
Solvent control	0	0	0	0
48	0	0	0	0
80	0	1[a,c]	5[a,e]	6[d,e]
130	2[a]	8[b,c]	10	10
220	7	10	10	10
360	10	10	10	10
600	10	10	10	10

[a] Several fish exhibited a complete loss in equilibrium.
[b] One fish exhibited a complete loss in equilibrium.
[c] One fish exhibited a partial loss in equilibrium.
[d] All fish exhibited a complete loss in equilibrium.
[e] All survivors exhibited darkened pigmentation.

Table A7.4.1.1-24: Effect data

Parameter	96 h [µg/L][1]	95 % c.l.
LC_{50}	75	48 to 130

[1] Based on nominal concentrations.

Table A7.4.1.1-25: Validity criteria for acute fish test according to OECD Guideline 203

	fulfilled	Not fullfilled
Mortality of control animals <10%	**Yes**	
Concentration of dissolved oxygen in all test vessels > 60% saturation	**No**	
Concentration of test substance ≥80% of initial concentration during test	**Not measured**	

Section A7.4.1.2-01 Annex Point IIA VII.7.2		Acute toxicity to invertebrates *Daphnia magna*	
		41 REFERENCE	**Official use only**
41.1	**Reference**	Xxxxxxxxxx, XX. and Xxxxxxxxxx, X. (XXXXx). Acute toxicity of LM-2219 to daphnids (*Daphnia magna*). XXXXXXXXXXXXXXXXXXXXXXXXX, laboratory report number XXXXXXXXXXXXXXXXX, XX Xxxxxxx XXXX (unpublished).	
41.2	**Data protection**	Yes.	
41.2.1	Data owner	LiphaTech S.A.S.	
41.2.2	Companies with letter of access	None.	
41.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		42 GUIDELINES AND QUALITY ASSURANCE	
42.1	**Guideline study**	Yes. ASTM (1980), comparable to OECD 202 (I) and EU C.2.	
42.2	**GLP**	Yes.	
42.3	**Deviations**	Yes. No analysis of test media for difethialone.	
		43 MATERIALS AND METHODS	
43.1	**Test material**	Difethialone (termed LM-2219 in the report).	
43.1.1	Lot/Batch number	XXXXXXXX.	
43.1.2	Purity	XX.X% (w/w).	
43.1.3	Further relevant properties	Photolysis half-life of difethialone is less than 1 hour, log octanol:water partition coefficient is 6.29 and water solubility is < 4.8 mg/L at 20°C.	x
43.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Stock solutions prepared with 1,000 µg/mL and 50 µg/mL in dimethylformamide. Stock solution then added to dilution water and stirred.	
43.3	**Reference substance**	No.	
43.4	**Testing procedure**		
43.4.1	Dilution water	See Table A7.4.1.2-2.	
43.4.2	Test organisms	See Table A7.4.1.2-3.	
43.4.3	Test system	See Table A7.4.1.2-4.	x
43.4.4	Test conditions	See Table A7.4.1.2-5.	
43.4.5	Duration of the test	48 hours.	
43.4.6	Test parameter	Immobilisation.	
43.4.7	Monitoring of TS concentration	No.	
43.4.8	Statistics	Non-linear interpolation.	

Section A7.4.1.2-01 **Annex Point IIA VII.7.2**		**Acute toxicity to invertebrates** ***Daphnia magna***	
		44 RESULTS	
44.1	**Results test substance**		
44.1.1	Initial concentrations of test substance	See Table A7.4.1.2-6.	
44.1.2	Effect data (Immobilisation)	See Table A7.4.1.2-6.	
44.1.3	Other effects	None reported.	
44.2	**Results of controls**	See Table A7.4.1.2-6.	
44.3	**Test with reference substance**	Not performed.	
		45 APPLICANT'S SUMMARY AND CONCLUSION	
45.1	**Materials and methods**	An acute static toxicity test with *D. magna* in accordance in principle with OCED 202 (I) and EC method C.2. The test substance was not analysed in the test media.	
45.2	**Results and discussion**		
45.2.1	EC_{50}	24-hour EC_{50} = > 5.0 µg/L 48-hour EC_{50} = 4.4 µg/L	x
45.3	**Conclusion**		
45.3.1	Reliability	2	
45.3.2	Deficiencies	Yes. No analysis of test media. However - refer to fish acute test.	
		Evaluation by Competent Authorities	
		Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
		EVALUATION BY RAPPORTEUR MEMBER STATE	
Date		13 December 2004; 31st October 2006	
Materials and Methods		Agree with applicant's summary and conclusion **Comments (3.1.3):** The water solubility of difethialone is set to be 0.39 mg/l. **Comments (3.4.3)**: Mistake in table A7.4.1.2-4 regarding amount of daphnia.: 20 animals per vessel and 4 vessels per concentration results in 80 animals/concentration. According to Doc IV 5 animals per vessel and 4 vessels/concentration (=20 animals/concentration) were used.	

Section A7.4.1.2-01 **Annex Point IIA VII.7.2**	**Acute toxicity to invertebrates** ***Daphnia magna***	
Results and discussion	Agree with applicant's version **Comments (5.2.1)**: The study is not fully valid as the test concentrations have not been verified analytically. Moreover, the static test design is not preferable for the testing of difficult substances like difethialone. The real exposure concentrations in test are likely to have slightly decreased over the test period mainly due to the low water solubility of difethialone and possibly due to adsorption of the test substance to glassware. The toxicity of difethialone to *Daphnia magna* might therefore be underestimated.	
Conclusion	Agree with applicant's version	
Reliability	2	
Acceptability	Acceptable with the restrictions noted above	
Remarks	Although nominal concentrations were not measured the test was performed as GLP study and difethialone has been shown to be stable in aqueous solution within the present timeframe of the test.	

Table A7.4.1.2-1: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Vehicle	Yes. Stock solution in dimethylformamide prepared at 1,000 µg/mL and a secondary stock at 50 µg/mL. Stock solutions then added directly to dilution water and stirred before use.
Concentration of vehicle	0.1 mL/L.
Vehicle control performed	Yes, 0.1 mL dimethylformamide/L.

Table A7.4.1.2-2: Dilution water

Criteria	Details
Source	Fortified well water (ASTM 1980) and filtered to remove organic contaminants.
Alkalinity	110 - 130 mg $CaCO_3$/L
Hardness	160 - 180 mg $CaCO_3$/L
pH	7.9 - 8.3
Oxygen content	> 60% ASV
Conductance	400 - 600 µmhos/cm
Holding water different from dilution water	No

Table A7.4.1.2-3: Test organisms

Criteria	Details
Source	Laboratory culture at XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Age	≤ 24 hours old at test start
Breeding method	Parthenogenic culture
Kind of food	Green algae and yeast suspension
Feeding frequency	Once daily
Pretreatment	None
Feeding of animals during test	No

Table A7.4.1.2-4: Test system

Criteria	Details
Renewal of test solution	Static
Volume of test vessels	200 mL
Volume/animal	10 mL
Number of animals/vessel	20
Number of vessels/ concentration	4

Table A7.4.1.2-5: Test conditions

Criteria	Details
Test temperature	21°C
Dissolved oxygen	84 - 90% ASV
pH	7.8 - 8.2
Adjustment of pH	No
Quality/Intensity of irradiation	Sylvania Growlux and Cool White fluorescent lights at 7 hectolux
Photoperiod	16 hours daily

Table A7.4.1.2-6: Immobilisation data

Nominal Test-Substance Concentration [µg/L]	Immobile *Daphnia* (%)	
	24 hours	48 hours
Control	0	0
Solvent control	0	0
0.40	0	0
0.65	0	0
1.1	0	0
1.8	0	0
3.0	0	0
5.0	45	70

Table A7.4.1.2-7: Effect data

Endpoint	EC_{50}[1]	95 % c.l.	EC_0[1,2]
24 h [µg/L]	> 5.0	> 3 (lower limit)	3.0
48 h [µg/L]	4.4	> 3 (lower limit)	3.0

[1] Based on nominal concentrations.
[2] Based on visual observation of data.

Table A7.4.1.2-8: Validity criteria for acute daphnia immobilistaion test according to OECD Guideline 202

	Fulfilled	Not fullfilled
Immobilisation of control animals <10%	**Yes**	
Control animals not staying at the surface	**Not reported**	
Concentration of dissolved oxygen in all test vessels >3 mg/l	**Yes**	
Concentration of test substance ≥80% of initial concentration during test	**Not analysed**	

Section A7.4.1.3-01 Annex Point IIA VII.7.3		Growth inhibition test on algae	
		46 REFERENCE	**Official use only**
46.1	**Reference**	Xxxxxxxxx, XX (XXXX). Difethialone: Toxicity to the green alga *Selenastrum capricornutum*. XXXXXXXXXXXXXXXXX., XXXXXXXXXXXXXXXXXXXXXX, laboratory report number XXXXXXX, XX Xxxxxxxx XXXX (unpublished).	
46.2	**Data protection**	Yes.	
46.2.1	Data owner	LiphaTech S.A.S.	
46.2.2	Companies with letter of access	None.	
46.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		47 GUIDELINES AND QUALITY ASSURANCE	
47.1	**Guideline study**	Yes. OECD 201 and EU method C.3.	
47.2	**GLP**	Yes.	
47.3	**Deviations**	No.	
		48 MATERIALS AND METHODS	
48.1	**Test material**	Difethialone.	
48.1.1	Lot/Batch number	XXXXXXX.	
48.1.2	Purity	XX.XX% (w/w).	
48.1.3	Further relevant properties	Photolysis half-life of difethialone is less than 1 hour, log octanol:water partition coefficient is 6.29 and water solubility is < 4.8 mg/L at 20°C.	x
48.1.4	Method of analysis	Aqueous samples analysed by HPLC (fluoresecence detector) either directly or diluted with acetonitrile.	
48.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Stock solution prepared in dimethylformamide (1.8 mg/mL). Stock solution added to algal medium and stirring vigorously.	
48.3	**Reference substance**	No.	
48.4	**Testing procedure**		
48.4.1	Culture medium	Composition based on that of Miller *et al.* (1978), EPA-600/9-78-018. $Na_2EDTA.2H2O$ added at 0.3 mg/L of culture medium.	
48.4.2	Test organisms	See Table A7.4.1.3-2.	
48.4.3	Test system	See Table A7.4.1.3-3.	
48.4.4	Test conditions	See Table A7.4.1.3-4.	
48.4.5	Duration of the test	72 hours.	
48.4.6	Test parameter	Inhibition of culture growth, measuring cell density areas under the	

Section A7.4.1.3-01 Annex Point IIA VII.7.3		Growth inhibition test on algae	
		growth curve and average specific growth rates.	
48.4.7	Sampling	Algal growth: 24, 48 and 72 hours. Medium pH at 0 and 72 hours. Temperature measured daily. Light intensity measured once only.	
48.4.8	Monitoring of TS concentration	Yes. See Table A7.4.1.3-6. Due to the limitations of the analytical method only the two highest test concentrations were analysed. Low recoveries were most likely due to the known photoinstability of difethialone.	x
48.4.9	Statistics	Weibull model.	
		49 RESULTS	
49.1	**Limit Test**	Not performed.	
49.2	**Results test substance**		
49.2.1	Initial concentrations of test substance	See Table A7.4.1.3-5.	
49.2.2	Actual concentrations of test substance	See Table A7.4.1.3-6.	x
49.2.3	Cell concentration data	See Table A7.4.1.3-5.	
49.2.4	Effect data (cell multiplication inhibition)	72-hour E_bC_{50} = 65 µg/L (95% confidence limits of 61 to 69 µg/L) 72-hour E_rC_{50} = > 180 µg/L 72-h NOE_bC = 18 µg/L 72-h NOE_rC = 32 µg/L	
49.3	**Results of controls**	See Table A7.4.1.3-5.	
49.4	**Test with reference substance**	Not performed.	
		50 APPLICANT'S SUMMARY AND CONCLUSION	
50.1	**Materials and methods**	Algal growth inhibition test with *S. capricornutum* in accordance with OECD 201 and EU method C.3.	
50.2	**Results and discussion**		
50.2.1	NOEC	72-h NOE_bC = 18 µg/L. 72-h NOE_rC = 32 µg/L.	
50.2.2	E_rC_{50}	72-hour E_rC_{50} = > 180 µg/L.	
50.2.3	E_bC_{50}	72-h E_bC_{50} = 65 µg/L.	
50.3	**Conclusion**	Validity criteria fulfilled.	
50.3.1	Reliability	1	x
50.3.2	Deficiencies	None.	x

Section A7.4.1.3-01 **Annex Point IIA VII.7.3**	**Growth inhibition test on algae**	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	13 December 2004; revised 31 October 2006	
Materials and Methods	Agree with applicant's version **Comment (3.1.3):** The water solubility of difethialone is set to be 0.39 mg/l.	
Results and discussion	Agree with applicant's summary and conclusion **Comment (4.2.2):** It should be stated that the results are based on nominal values. Only the two highest test concentrations were measured analytically. However, no measurable concentrations were found. This may be a procedure problem for example caused by adherence to glassware as noted in other tests or uptake by algae. The known photoinstability of difethialone may be another reason. **Comment (3.4.7):** A maximum increase of 3.2 pH units was observed during the test. At the start of the test the pH was 7.4 and ranged from 8.0 to 10.6 at the end of the test. **Comment (3.4.8):** The measurement of the concentrations was conducted in an external stability control, not in the test media with algae itself. This stability control had been stored deep-frozen for 16 month before analysing it. As it can be seen in table A7.4.1.3-6 concentrations of difethialone were below detection limit both at time 0 hours and 72 hours. Therefore these measurements can not be regarded representative for this test. **Comment (5.3.1 and 5.3.2):** As difethialone is photo-labile and adsorbs to glassware and probably also to algae, it can be assumed that exposure concentrations are considerably lower than nominal concentrations. No monitoring of test substance concentration has been conducted during the test. For a substance like difethialone it seems not possible to conduct an algae test without real analytical measurements which would allow correcting the results for recovery. Therefore the reliability indicator will be changed to 3.	
Conclusion	Agree with applicant's version	
Reliability	Due to the restrictions stated above, the reliability should be changed from 1 to 3.	
Acceptability	Acceptable with the restrictions noted above	
Remarks		

Table A7.4.1.3-1: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Vehicle	Yes, dimethylformamide
Concentration of vehicle	100 µL/L
Vehicle control performed	Yes, 100 µL dimethylformamide/L

Table A7.4.1.3-2: Test organisms

Criteria	Details
Species	*Selenastrum capricornutum*
Strain	ATCC 22662
Laboratory culture	Yes
Method of cultivation	Axenic laboratory culture
Initial cell concentration	1.04×10^4 cells/mL

Table A7.4.1.3-3: Test system

Criteria	Details
Volume of culture flasks	250 mL
Culturing apparatus	Glass conical flasks held in a temperature-controlled orbital shaker
Light quality	Cool-white illumination, 7,550 lux (90.5 µE/m^2/s)
Procedure for suspending algae	Shaking at 160 rpm
Number of vessels/ concentration	3 with 6 replicates for the solvent control

Table A7.4.1.3-4: Test conditions

Criteria	Details
Test temperature	24.1 - 24.2 °C
pH	7.4 at start to 8.0 - 10.6 at end
Light intensity	7,550 lux
Photoperiod	Continuous

Table A7.4.1.3-5: Algal growth

Nominal Test-Substance Concentration [µg/l]	Algal cell density (× 10^4 cells/mL)			Mean areas under the growth curve		Mean growth rates	
	24 h	48 h	72 h	0-72 h	% of solvent control	0-72 h	% of solvent control
Control	5.15	44.5	275	184.8	105	1.860	101
Solvent control	5.12	43.9	260	176.5	-	1.841	-
5.6	5.31	41.0	232	159.5	90	1.802	98
10	4.56	35.3	237	155.6	88	1.809	98
18	4.84	38.9	238	160.1	91	1.811	98
32	4.26	29.0	191	126.4*	72	1.738	94
56	4.40	25.1	144	99.0*	56	1.644*	89
100	4.08	15.7	54.0	44.2*	25	1.316*	72
180	3.80	15.0	43.7	38.0*	22	1.246*	68

* Significantly different to the solvent control at $p < 0.05$.

Table A7.4.1.3-6: Analytical results

Nominal concentration of difethialone [µg/L]	Measured concentration (µg/L)[1]		Mean measured concentration (µg/L)	Mean measured concentration as % of nominal (%)
	0 h	72 h		
100	< 67	< 67	< 67	< 67
180	< 130	< 130	< 130	< 72

[1] Limit of detection was 130 µg/L, differing between the two samples due to dilution (× 2) of the 180 µg/L sample before analysis.

Table A7.4.1.3-7: Validity criteria for algal growth inhibition test according to OECD Guideline 201

	Fulfilled	Not fullfilled
Cell concentration in control cultures increased at least by a factor of 16 within 3 days	**Yes**	
Concentration of test substance ≥80% of initial concentration during test	**No**	

Section A7.5.1.2-01 Annex Point IIIA XIII.3.2	Earthworm, acute toxicity test	
	51 REFERENCE	**Official use only**
51.1 Reference	Xxxxxx, xx and Xxxxxxxx, X (XXXX). Difethialone: Determination of acute toxicity (LC50) to earthworms. Xxxxxxxxxxxxxxxx, laboratory report number XXXXXX, XX Xxxxxxxx XXXX (unpublished).	
51.2 Data protection	Yes.	
51.2.1 Data owner	LiphaTech S.A.S.	
51.2.2 Companies with letter of access	None.	
51.2.3 Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
	52 GUIDELINES AND QUALITY ASSURANCE	
52.1 Guideline study	Yes. OECD 207 (1984).	
52.2 GLP	Yes.	
52.3 Deviations	No.	
	53 METHOD	
53.1 Test material	Difethialone.	
53.1.1 Lot/Batch number	XXXXXXX (synonymous with XXXXXXXXXXX).	
53.1.2 Purity	XX.XX% (w/w).	
53.1.3 Further relevant properties	Aqueous solubility limit < 4.8 mg/L; Vapour pressure: < 1.3×10^{-5} Pa Soil DT_{50}: 204 days	x

Soil type	Adsorption Kd ($\times 10^5$)	Adsorption Koc ($\times 10^5$)
clay	88	6,200
sand	2.3	1,000
sandy clay loam	240	53,000
sandy loam	7.3	2,700

53.1.4 Method of analysis	Not appropriate.	
53.2 Reference substance	2-chloroacetamide (separate study).	
53.3 Testing procedure		
53.3.1 Preparation of the test substance	See Table A7.5.1.2-1.	
53.3.2 Application of the test substance	See Section 5.1 and Table A7.5.1.2-1.	
53.3.3 Test organisms	See Table A7.5.1.2-2.	

Section A7.5.1.2-01 **Annex Point IIIA XIII.3.2**		**Earthworm, acute toxicity test**	
53.3.4	Test system	See Table A7.5.1.2-3.	
53.3.5	Test conditions	See Table A7.5.1.2-4.	
53.3.6	Test duration	14 days.	
53.3.7	Test parameter	Mortality and sub-lethal effects (bodyweight and behaviour).	
53.3.8	Examination	Daily checks for live and/or dead worms at soil surface. Overall mortality assessments by searching through soil from each test vessel on days 7 and 14. Individual bodyweight measurements on days 0 and 14.	
53.3.9	Monitoring of test substance concentration	No.	
53.3.10	Statistics	NOEC (survival) estimated by Fisher's Exact Test; Significant bodyweight changes estimated by Dunnett's t-test.	
		54 RESULTS	
54.1	**Soil test**		
54.1.1	Initial concentrations of test substance	0 (control and solvent control), 62.5, 125, 250, 500 and 1,000 mg/kg dry artificial soil (definitive test, based on results of range-finder).	
54.1.2	Effect data (Mortality)	Mortality data and endpoints are summarised in Table A7.5.1.2-5 and Table A7.5.1.2-6.	
54.1.3	Other effects	Data showing impact on bodyweight are summarised in Table A7.5.1.2-8. No other effects were observed.	
54.2	**Results of controls**		
54.2.1	Mortality	See Table A7.5.1.2-5.	
54.2.2	Number/ percentage of earthworms showing adverse effects	See Table A7.5.1.2-8.	
54.3	**Test with reference substance**	Performed as separate study.	
54.3.1	Concentrations	12, 19.5, 31.3, 50 and 80 mg 2-chloroacetamide/kg.	
54.3.2	Results	60 mg/kg (with 95% confidence limits of 56 to 65 mg/kg).	
		55 APPLICANT'S SUMMARY AND CONCLUSION	
55.1	**Materials and methods**	The 14-day acute toxicity of difethialone to the earthworm (*Eisenia foetida*) was determined in the laboratory according to OECD Guideline 207. Worms were exposed over a period of 14 days to soil-incorporated difethialone at nominal concentrations of 0 (control: artificial soil only, comprising 70% industrial sand, 20% kaolin clay, 10% sphagnum peat, moisture content: approximately 35% of dry weight, and solvent control: as control, but with a portion of the sand combined with chloroform that was evaporated prior to mixing), 62.5, 125, 250, 500 and 1,000 mg/kg dry soil. Solutions of difethialone dissolved in chloroform were mixed with portions of a sand carrier and the organic solvent removed by evaporation (3 hours). Sand portions plated with	

Section A7.5.1.2-01 Annex Point IIIA XIII.3.2	Earthworm, acute toxicity test	
	various amounts of test substance were then thoroughly mixed into the appropriate soil batches. There were four 1 L glass beakers, each containing 750 g soil, per treatment. A weighed group of ten worms (at least two months old, clitellated) was placed on the soil surface in each container. The test vessels were incubated under continuous lighting at a temperature range of 20 to 22°C. Daily checks were made for worms on the soil surface. Numbers of dead and surviving worms were counted on day 7. Final counts were made on day 14, when the live worms retrieved from each container were also weighed. Some initial pH values in the definitive test, including that recorded for the solvent control treatment, were below the recommended Guideline range (Table A7.5.1.2-4). These measurements varied widely and randomly and did not correlate with a set of in-range readings taken during an earlier range-finder test. It is likely, therefore, that the outlier measurements were erroneous. In any case, no mortalities or adverse effects occurred in the solvent control, indicating that these deviations had no influence on the outcome of the test.	
55.2 Results and discussion	Biological effects were assessed in relation to nominal difethialone concentrations. By the end of the 14-day test period there were no mortalities in the solvent control treatment or at 62.5 mg/kg dry soil. Mortality was 5, 5, 2.5, 7.5 and 22.5% in the untreated control and at 125, 250, 500 and 1,000 mg/kg, respectively. Only the mortality recorded at 1,000 mg/kg was statistically significant ($p < 0.05$), compared to the solvent control (Table A7.5.1.2-5). The NOEC with respect to survival was therefore 500 mg/kg. Earthworms of all treatment groups burrowed beneath the soil surface within 30 minutes of the beginning of the test. After 14 days exposure, slight mean weight gains were recorded in both the control treatments, in contrast to the groups of earthworms exposed to the test substance, where slight, though statistically significant ($p < 0.05$), weight reductions occurred at all difethialone concentrations (Table A7.5.1.2-8). However, this effect showed no clear dose-response relationship. There were no other abnormal behavioural or pathological effects. Difethialone is neither volatile nor rapidly degraded in soil. Volatilisation from and degradation in the test system may therefore be ruled out as factors contributing to the low acute toxicity of difethialone to earthworms. Difethialone adsorbs strongly to soil and organic matter and it is possible that this behaviour influenced the outcome of the test.	
55.2.1 LC_0 (NOEC)	500 mg/kg dry soil.	
55.2.2 LC_{50}	> 1,000 mg/kg dry soil.	
55.2.3 LC_{100}	> 1,000 mg/kg dry soil.	
55.3 Conclusion	The guideline validity criterion was fulfilled (Table A7.5.1.2-7). No significant mortality occurred at difethialone concentrations ranging from 62.5 to 500 mg/kg dry soil and significant mortality was confined to the 1,000 mg/kg dry soil treatment, the highest concentration tested. The 14-day acute LC_{50} of difethialone to earthworms was therefore greater than 1,000 mg/kg and the survival NOEC was 500 mg/kg.	x
55.3.1 Other Conclusions	None.	
55.3.2 Reliability	1.	
55.3.3 Deficiencies	None.	

Section A7.5.1.2-01 **Annex Point IIIA XIII.3.2**	**Earthworm, acute toxicity test**	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	4 January 2005, revised 20 December 2006	
Materials and Methods	**Comment (3.1):** The water solubility of difethialone is 0.39 mg/l. The soil refers to reference 7.1.3-01. Agree with applicant's version	
Results and discussion	Agree with applicant's version	
Conclusion	**Comment (5.3):** The guideline validity criterion was fulfilled (Table A7.5.1.2-7). No significant mortality occurred at difethialone concentrations ranging from 62.5 to 500 mg/kg dry soil and significant mortality was confined to the 1,000 mg/kg dry soil treatment, the highest concentration tested. The 14-day acute LC_{50} of difethialone to earthworms was therefore greater than 1,000 mg/kg. All treatments showed reduced weight during test in contrast to a weight gain in the controls. It can be assumed that continued exposure would eventually lead to more mortality due to starvation. However, no dose-effect relationship for the endpoint bodyweight could be obsverved and hence no L/EC50 could be derived. As this is an acute test the guideline does not allow for a NOEC derivation based on weight loss.	
Reliability	1	
Acceptability	Acceptable	
Remarks	As the TGD express PEC/PNEC on a wwt basis the results have to be converted taking into account a standard conversion factor from EUSES of 1.13.	

Table A7.5.1.2-1: Preparation of TS solution

In case of the use of an organic solvent	
Dispersion	No.
Vehicle	Yes. Difethialone dissolved in chloroform was plated on a sand carrier by evaporation of the organic solvent.
Concentration of vehicle	90 mL chloroform/150 g sand/3 kg soil batch (definitive test).
Vehicle control performed	Yes. 90 mL chloroform/150 g sand/3 kg soil batch (definitive test).
Other procedures	None.

Table A7.5.1.2-2: Test organisms

Criteria	**Details**
Species/strain	*Eisenia foetida foetida*.
Source of the initial stock	Blades Biological, UK.
Culturing techniques	Not reported. Cultures maintained by supplier, separate batches obtained for range-finder and definitive test.
Age/weight	Age at least two months (clitellated), weight range 300 to 600 mg.
Pre-treatment	Acclimatised for at least seven days to controlled-temperature laboratory conditions.

Table A7.5.1.2-3: Test system

Criteria	Details
Artificial soil test substrate	Approximate composition: 70% industrial sand, 20% kaolinite, 10% sphagnum peat. $CaCO_3$ added (approximately 0.5% of total dry weight) to adjust pH to 6 ± 0.5. Contents blended with deionised water to give a nominal moisture content of 35%. Total and organic carbon content not reported.
Test mixture	3 kg soil batches mixed with 187.5, 375.1, 750.2, 1,500.5 and 3,000.3 g difethialone to give nominal concentrations of 62.5, 125, 250, 500 and 1,000 mg/kg, respectively.
Size, volume and material of test container	1 L glass beakers covered with perforated plastic film.
Amount of artificial soil (kg)/ container	ca. 750 g wet weight.
Nominal test concentrations	0 (control and solvent control), 1, 10, 100 and 1,000 mg/kg dry artificial soil (range-finder); 0 (control and solvent control), 62.5, 125, 250, 500 and 1,000 mg/kg dry artificial soil (definitive test).
Number of replicates/concentration	4 (definitive test).
Number of earthworms/test concentration	40 (definitive test).
Number of earthworms/container	10
Light source	Fluorescent lighting.

Table A7.5.1.2-4: Test conditions

<table>
<tr><th>Criteria</th><th colspan="3">Details</th></tr>
<tr><td>Test temperature</td><td colspan="3">21 ± 1°C.</td></tr>
<tr><td rowspan="10">Moisture content</td><td rowspan="2">Nominal treatment (mg difethialone/kg dry soil)</td><td colspan="2">Moisture content</td></tr>
<tr><td>day 0[1]</td><td>day 14[2]</td></tr>
<tr><td>Control</td><td>34</td><td>32 – 34</td></tr>
<tr><td>Solvent control</td><td>35</td><td>32 – 33</td></tr>
<tr><td>62.5</td><td>33</td><td>32 – 33</td></tr>
<tr><td>125</td><td>34</td><td>32 – 34</td></tr>
<tr><td>250</td><td>35</td><td>33 – 34</td></tr>
<tr><td>500</td><td>35</td><td>32 – 34</td></tr>
<tr><td>1,000</td><td>34</td><td>31 – 33</td></tr>
<tr><td colspan="3">[1] single determination on bulk sample;
[2] range of determinations on four replicate vessels.</td></tr>
<tr><td rowspan="13">pH</td><td rowspan="2">Nominal treatment (mg difethialone/kg dry soil)</td><td colspan="2">Initial pH</td></tr>
<tr><td>rangefinder</td><td>definitive</td></tr>
<tr><td>Control</td><td>5.55</td><td>6.30</td></tr>
<tr><td>Solvent control</td><td>5.50</td><td>5.14*</td></tr>
<tr><td>1</td><td>5.61</td><td>-</td></tr>
<tr><td>10</td><td>5.52</td><td>-</td></tr>
<tr><td>62.5</td><td>-</td><td>5.56</td></tr>
<tr><td>100</td><td>5.65</td><td>-</td></tr>
<tr><td>125</td><td>-</td><td>5.32*</td></tr>
<tr><td>250</td><td>-</td><td>5.16*</td></tr>
<tr><td>500</td><td>-</td><td>4.78*</td></tr>
<tr><td>1,000</td><td>5.57</td><td>5.13*</td></tr>
<tr><td colspan="3">* pH value outside Guideline limits. (Compared to the range-finder data set, the definitive test values show large, random variation, suggesting measurement errors in the latter test).</td></tr>
<tr><td>Adjustment of pH</td><td colspan="3">No.</td></tr>
<tr><td>Light intensity / photoperiod</td><td colspan="3">Intensity not reported. Photoperiod continuous.</td></tr>
<tr><td>Relevant degradation products</td><td colspan="3">None.</td></tr>
</table>

Table A7.5.1.2-5: Mortality data

Nominal test cubstance concentration [mg/kg artificial soil]	Cumulative mortality			
	Number (out of 40/treatment)		Percentage	
	7 d	14 d	7 d	14 d
Control	1	2	2.5	5.0
Solvent control	0	0	0.0	0.0
62.5	0	0	0.0	0.0
125	2	2	5.0	5.0
250	1	1	2.5	2.5
500	2	3	5.0	7.5
1,000	3	9	7.5	22.5*

* significantly higher than the solvent control ($p < 0.05$, Fisher's Exact Test).

Table A7.5.1.2-6: Effect data

Endpoint	14 d [mg/kg soil]	95 % c.l.
NOEC (survival)	500 (n)	n.a.
LC_0	-	-
LC_{50}	> 1,000 (n)	n.a.
LC_{100}	> 1,000 (n)	n.a.

(n): nominal concentrations;
n.a.: not applicable.

Table A7.5.1.2-7: Validity criteria for acute earthworm test according to OECD 207

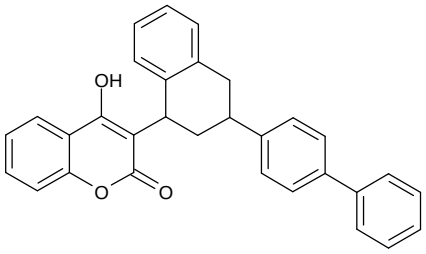
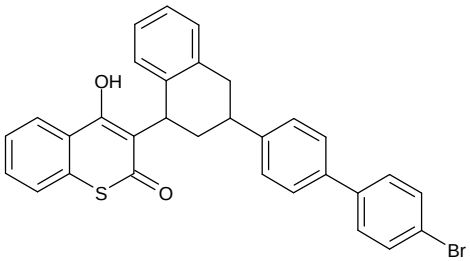
	Fulfilled	Not fulfilled
Mortality of control animals < 10%	Yes	-

Table A7.5.1.2-8: Bodyweight data

Nominal test substance concentration [mg/kg artificial soil]	Mean bodyweight (g)								Overall mean bodyweight change (%)
	0 d				14 d				
	rep 1	rep 2	rep 3	rep 4	rep 1	rep 2	rep 3	rep 4	
Control	0.422	0.405	0.425	0.432	0.437	0.413	0.466	0.459	+ 5.4
Solvent control	0.399	0.430	0.334	0.431	0.441	0.441	0.374	0.443	+ 7.0
62.5	0.417	0.410	0.395	0.418	0.441	0.390	0.399	0.403	- 2.2*
125	0.417	0.426	0.419	0.403	0.417	0.401	0.404	0.405	- 2.3*
250	0.433	0.420	0.379	0.404	0.440	0.416	0.377	0.393	- 0.7*
500	0.422	0.422	0.354	0.415	0.404	0.425	0.360	0.401	- 1.3*
1,000	0.421	0.385	0.404	0.430	0.384	0.360	0.398	0.418	- 4.9*

* significantly reduced compared to the solvent control ($p < 0.05$, Dunnett's t-Test).

Section 7.5.3.1.3-03 Annex Point IIIA XIII.1.3		Effects on reproduction of birds	
		56 REFERENCE	**Official use only**
56.1	**Reference**	Xxxxxx, X (2006). Avian reproduction study with difenacoum in the Japanese quail (*Coturnix coturnix japonica*). XXXXXXXXXXXXXXXXXXXXXXX Laboratory report number XXXXX, XX Xxxxx XXXX (unpublished).	
56.2	**Data protection**	Yes.	
56.2.1	Data owner	XXXXXXXXXXXXX	
56.2.2	Companies with letter of access	Liphatech S.A.S., XXXXXXXXXXXXXX	
56.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I.	
		57 GUIDELINES AND QUALITY ASSURANCE	
57.1	**Guideline study**	Yes. OECD Test Guideline 206: Avian Reproduction Test, 1984, with modifications to meet requirements of OECD Test Guideline "Draft Document 1998": Avian Toxicity Test in the Japanese Quail or Bobwhite Quail, and USEPA Ecological Effects Guideline OPPTS 850.2300: Avian Reproduction Test.	
57.2	**GLP**	Yes.	
57.3	**Deviations**	Mortality (12.5%) slightly exceeded 10% in the vehicle control (VC) group, but was attributed to pen-mate aggression and was therefore considered not to impact upon the validity of the study. There were some excursions of temperature and humidity with respect to the ranges recommended by OECD Guideline 206 and the OECD draft revision. During the storage of eggs the humidity was outside the recommended range of 55-75% for most of the time. The temperature in the hatching room was mostly outside the recommended range and humidity was below the OECD recommendation. However, viable embryos, live embryos and hatching success were 94.4%, 95.3% and 83.5%, respectively, in the vehicle control (VC) group and there is consequently no evidence that these excursions had any adverse imact on embryo development.	
		58 METHOD	
58.1	**Test material**	Difenacoum. Difenacoum is structurally closely related to difethialone (see below) and is an antivitamin K (AvK) anticoagulant rodenticide with a similar mode of action. Therefore, this study is considered to be relevant to difethialone in qualitative terms, in that the nature of the toxic effects is expected to be similar for all rodenticides with this mode of action, although the dietary concentrations at which effects are observed may differ.	x

Section 7.5.3.1.3-03 **Annex Point IIIA XIII.1.3**		**Effects on reproduction of birds**	
		difenacoum  	
		difethialone  	
58.1.1	Lot/Batch number	XXXXXXXX.	
58.1.2	Purity	XX.X% (w/w).	
58.1.3	Method of analysis	The extraction of difenacoum from avian feed involved homogenisation with acetone followed by evaporation. The extract was then further cleaned up using a hexane:acetonitrile liquid-liquid partition. An aliquot of the acetonitrile phase was taken for quantification by LC-MS/MS using positive ion chemical ionisation. A validated LOQ of 0.01 mg/kg difenacoum was obtained.	
58.2	**Administration of the test substance**	Incorporated in diet. See Table A7.5.3.1.3-11.	
58.3	**Testing procedure**		
58.3.1	Test organisms	See Table A7.5.3.1.3-12.	
58.3.2	Test system	See Table A7.5.3.1.3-13.	
58.3.3	Diet	"5639 Mazuri® Exotic Gamebird Breeder" containing 0.64 ppm vitamin K (as menadione dimethylpyrimidinol bisulfite) was used as the basal feed to prepare all test diets administered to the adult birds. The test substance was dissolved in HPLC-grade acetone to make a stock solution. For each dietary concentration, an appropriate aliquot of the stock solution was transferred to another container and diluted with additional acetone. The total amount of vehicle added to a batch was set at two percent by weight. The final solution for each dietary level was added to the basal feed in the mixing bowl of a large Hobart mixer. The diet was mixed for 15 minutes after the vehicle was added. The Vehicle Control (VC) diet was always mixed first with neat acetone, followed by the T1, T2, T3 and T4 test diets. Fresh test diets were prepared at least every two weeks. Prepared diets were stored in a walk-in freezer for two weeks, at which time a new batch was mixed. Unamended "5637 Mazuri® Exotic Gamebird Starter" containing 0.81 ppm vitamin K (as menadione dimethylpyrimidinol bisulfite) was fed to the first generation hatchlings.	

Section 7.5.3.1.3-03 **Annex Point IIIA XIII.1.3**	**Effects on reproduction of birds**	
58.3.4 Test conditions	See Table A7.5.3.1.3-14.	
58.3.5 Duration of the test	Adult Treatment Period: 10 weeks pre-egg laying; 10 weeks egg-laying. Hatchling Observation Period: 14 days post-hatch.	
58.3.6 Test parameters	Adult Parameters: Daily observations, diet consumption, body weight, necropsy including wet weights of the liver, spleen and testes. Reproductive Parameters: Eggs laid, eggshell thickness, defective and cracked eggs, viable embryos, live embryos. Hatchling Parameters: Hatching success/hatchability, hatchling survival, hatchling body weights.	
58.3.7 Examination / Observation	The birds were observed daily during the 20 week exposure period. Inspections were made to monitor symptoms that may be indicative of test substance related effects. Birds that died during the treatment period were removed, weighed and necropsied. Feed consumption of each pair of birds was measured weekly during the exposure period. The body weight of each bird was measured at the initiation of the 14-day acclimation period, on day 0, at the end of week 8, and at the end of week 20. At the conclusion of the treatment period, remaining birds were euthanized and necropsied for gross pathological abnormalities. Specific examination was made on the gastro-intestinal tract, liver, kidneys, bile duct, heart, spleen, and reproductive organs. Wet weights of the liver, spleen and testes were measured at the time of necropsy. Other observations were recorded as necessary.	

Section 7.5.3.1.3-03 **Annex Point IIIA XIII.1.3**	**Effects on reproduction of birds**	
58.3.8 Statistics	Adult endpoints and reproductive parameters were statistically analyzed using TOXSTAT Version 3.4. The experimental unit was each pen (or adult pair), except in the case of adult body weight, in which case the experimental unit was each adult bird. If a data set passed the chi-square test for normal distribution, and Bartlett's test for homogeneity of variance, it was analyzed by ANOVA. If no significant difference was identified by the ANOVA, no additional data was used. If ANOVA identified a difference, then the *post hoc* results generated by TOXSTAT were used. Bonferroni's test was used for pair-wise comparisons of each treatment with the control group. Bonferroni's test is appropriate when the replicates per group were not equal, as was the case in with many of the data sets. Data sets consisting of count data which did not pass the chi-square test and/or Bartlett's test, were transformed and analyzed again. If an appropriate transformation did not succeed in normalizing the distribution, or if the variance was not homogeneous, the original, untransformed data was analyzed by Kruskal-Wallis' non-parametric test (H-statistic). If a *post hoc* pair-wise comparison was indicated, Dunn's multiple comparison procedure was used. Dunn's procedure compares all possible pairs of means. If no significant difference was identified by the Kruskal-Wallis' test, no additional data was used. If the Kruskal-Wallis' test identified a significant difference, then the post hoc results were reported. Proportional (percentage) data were analyzed following the above process, but if the untransformed data failed normality and/or homogeneity tests, the data were transformed with "anscombe arcsin" or "arcsine (square root (Y))", and the appropriate test was performed (Kruskal-Wallis' or ANOVA), regardless of the results of the transformed analysis. Untransformed percent data that did not pass the normality and homogeneity tests were transformed. Depending upon the results of the transformation, the appropriate analysis of variance procedure was performed. Power analyses were performed for each test parameter to determine the probability of rejecting the null hypothesis of equal means (H_0), when in fact the alternative hypothesis of significantly different means is true (H_1). Re-stated, the power of the test is the probability of detecting a difference when there is a difference. The analysis is a pair-wise comparison of two means. In all cases, the mean values tested were the vehicle control group (VC) and the highest dietary concentration group, treatment level 4 (T4). The rationale for this comparison was that any test substance related effect would be expressed most strongly in the highest dose group. Power analyses were performed using the program XLStatistics. The test parameters were set at: Significance Level (α): 0.05 Test Hypotheses: H_0: $\mu_1-\mu_2 = 0$ H_1: $\mu_1-\mu_2 \neq 0$ (two-tailed test) Actual standard deviations associated with the means were used since the analyses were performed post hoc. The power statistic is expressed as $(1-\beta)$.	
	59 RESULTS	
59.1 Range finding test	Not performed.	

Section 7.5.3.1.3-03 **Annex Point IIIA XIII.1.3**		**Effects on reproduction of birds**	
59.1.1	Concentration	Not applicable.	
59.1.2	Number/ percentage of animals showing adverse effects	Not applicable.	
59.1.3	Nature of adverse effects	Not applicable.	
59.2	**Results test substance**		
59.2.1	Applied concentrations	Nominal Dietary Concentrations: 0 (VC), 0.001 (T1), 0.005 (T2), 0.020 (T3), 0.100 (T4) mg/kg diet, equivalent to: 0 (VC), 0.016 (T1), 0.075 (T2), 0.317 (T3), 1.642 (T4) mg/kg bw at the conclusion of the 20 week treatment period.	
59.2.2	Effect data (Mortality and reproductivity)	Adverse effects observed in adult birds during treatment period:	

Group	Nominal Concentration (mg a.i./kg diet)	Birds Euthanized [a]	Birds Died [b]	Week(s) Found Dead	Initial Number of Birds	Dead (%)	Group Observations
VC	0	9	5	5,7,9,14	40	12.5	Feather loss (head,back), pecking (head), abrasion (head,ear,eye,foot), found dead, and sacrificed.
T1	0.001	5	3	10,11,16	38	7.9	Feather loss (head,back,neck), pecking (head), abrasion (head), hemmorhage (beak). Subdermal hematoma (head), sacrificed, growth on foot, found dead.
T2	0.005	7	2	12,20	40	5.0	Feather loss (head, eye, neck, back), pecking (head), abscess (beak), abrasion (head,foot), sacrificed, subdermal hematoma (head), ataxic, growth on beak, injured (right leg), found dead.
T3	0.020	5	1	7	40	2.5	Feather loss (head,neck), pecking (head), abscess (head), abrasion(foot), hypo-reactivity, abrasion healing, feathers growing, found dead, sacrificed, wing drop, injured (wing), growth on beak, subdermal hematoma (head).
T4	0.100	7	1	16	40	2.5	Feather loss (head, back), pecking (head) abrasion (head,foot), sacrificed, ataxic, fluffed feathers, found dead, growth on beak.

[a] Single birds were euthanized if their pen-mate had died. Both members of a pair were euthanized if the pair was incompatible, described as repeated or routine agonistic behavior which was resulting in severe injury to one or both members of the pair. Both members of some pairs were also euthanized if they met the criterion for excessively low egg production in week 13 of the test. The criterion was egg production in week 13 which was less than or equal to two standard deviations below the mean egg production of the VC group in week 13.
[b] Includes only those birds that were found dead during the 20 week adult observation period. Not included are any birds euthanized during the test.

Reproduction parameters:

Section 7.5.3.1.3-03 **Annex Point IIIA XIII.1.3**	**Effects on reproduction of birds**	

Group	Nominal Conc. (mg a.i./ kg diet)	Egg Data							
		Eggs Laid	Mean Eggs /Hen /Week	Mean Hatchlings /Hen /Week	Mean Shell Thickness (mm)	Cracked Eggs (%) [a]	Viable Embryo (%) [b]	Live Embryo (%) [c]	Hatch (%) [d]
VC	0	889	6.5	4.0	0.215	9.8	94.4	95.3	83.5
T1	0.001	973	6.4	3.7	0.207	13.4	94.9	96.2	81.9
T2	0.005	963	6.1	3.2	0.216	10.0	87.2	95.2	77.0
T3	0.020	1056	6.2	4.0	0.215	7.7	95.5	96.2	81.4
T4	0.100	1048	6.4	3.8	0.213	9.9	90.7	97.5	83.0

[a] Percent Cracked Eggs = (cracked eggs/eggs candled) * 100.
[b] Percent Viable embryos = (viable embryos/eggs set) * 100.
[c] Percent Live Embryos = (live embryos/viable embryos) * 100.
[d] Percent Hatch = (hatchlings/viable embryos) * 100.

Refer also to table A7.5.3.1.3-15.

NOEC (No Observed Effect Concentration): Greater than the T4 dietary concentration:

0.100 mg/kg diet administered for 20 weeks.

NOEL (No Observed Effect Level): Greater than the T4 ingestion level:

0.01138 mg/kg body weight/day (mean males and females).

59.2.3 Body weight

Adults:

Group	Nominal Concentration (mg a.i./kg diet)	Mean Body Weight (g)		
		Week 0 [a, b]	Week 8 [c]	Week 20 [d]
		Male		
VC	0	211	236	273
T1	0.001	214	251	290
T2	0.005	214	251	284
T3	0.020	205	238	275
T4	0.100	202	234	266
		Female		
VC	0	225	278	304
T1	0.001	224	284	312
T2	0.005	229	279	315
T3	0.020	227	280	302
T4	0.100	231	291	318

[a] Differences in initial body weights (male and female) among groups were not significant when analyzed using ANOVA (F = 2.45, calculated F = 0.176).
[b] Differences in initial male body weights among groups were not significant when analyzed using ANOVA (F = 2.53, calculated F = 1.974). Differences in initial female body weights among groups were not significant when analyzed using ANOVA (F = 2.53, calculated F = 0.335).
[c] Differences in week 8 male body weights among groups were not significant when analyzed using ANOVA (F = 2.53, calculated F = 1.976). Differences in week 8 female body weights among groups were not significant when analyzed using ANOVA (F = 2.53, calculated F = 0.628).
[d] Differences in week 20 male body weights among groups were not significant when analyzed using ANOVA (F = 2.53, calculated F = 1.852). Differences in week 20 female body weights among groups were not significant when analyzed using ANOVA (F = 2.53, calculated F = 0.924).

Hatchlings:

Section 7.5.3.1.3-03
Annex Point IIIA XIII.1.3

Effects on reproduction of birds

Group	Nominal Concentration (mg a.i./kg diet)	Mean Day 0 Body Weight (g) [a]	Mean Day 14 Body Weight (g) [b]
VC	0	10	68
T1	0.001	10	67
T2	0.005	9	69
T3	0.020	9	59
T4	0.100	10	65

[a] Differences in mean day 0 body weight among groups were not significant when analyzed using Kruskal-Wallis (H = 9.490, calculated H = 2.121),
[b] Differences in mean day 14 body weight among groups were significant when analyzed using Kruskal-Wallis (H = 9.490, calculated H = 29.933), and a *post hoc* test using Dunn's multiple comparison test show T3 being significantly different than all other groups while all other groups, excluding T3, were not significantly different from each other.

59.2.4 Food consumption

Adults:

Mean Feed Consumption of Adult *Coturnix*
During the Avian Reproduction Test With Difenacoum

Group	Nominal Concentration (mg a.i./kg diet)	Feed Consumption (grams/bird/day) Week 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Mean[a]
VC	0	28	29	26	26	28	26	26	28	30	31	35	36	35	36	36	37	36	37	34	35	32
T1	0.001	28	30	27	28	31	29	29	31	32	34	35	37	37	38	38	38	37	39	36	37	34
T2	0.005	28	30	26	27	30	27	28	29	29	31	33	36	34	36	34	37	35	37	35	36	32
T3	0.020	28	30	27	27	31	27	27	29	30	31	34	36	36	37	37	38	36	36	36	38	33
T4	0.100	26	30	27	27	29	27	28	30	32	34	34	37	36	38	36	38	37	37	37	39	33

[a] Difference in mean feed consumption among groups were not significant when analyzed using ANOVA (F = 2.53, calculated F = 1.182).

59.2.5 Results of residue analysis

Batch #	Date Prepared	Mean Measured Concentration (mg a.i./kg diet) VC (0.000 mg a.i./kg diet)	T1 (0.001 mg a.i./kg diet)	T2 (0.005 mg a.i./kg diet)	T3 (0.020 mg a.i./kg diet)	T4 (0.100 mg a.i./kg diet)
1	May 9, 2005	-	-	-	0.0177	0.1017
2	June 7, 2005	-	-	-	0.0225	0.1295
11	September 19, 2005	-	-	-	0.0198	0.0845
Mean Measured Concentration		-	-	-	0.0200	0.1052
Standard Deviation		-	-	-	0.0024	0.0227
Percent of Nominal		-	-	-	100.0	105.2

T1 and T2 diets were not analysed as the concentration is below the LOQ of the validated analytical method. However, the lower dietary concentrations are verified indirectly firstly by the careful dilution of the stock solution, secondly by the consistent mixing process used to prepare all levels, and thirdly, by the analytical verification of difenacoum levels in the T3 and T4 diets, which were mixed in the same manner and at the same time.

59.2.6 Other effects

Birds that died during the treatment were necropsied and the significant findings included:

Section 7.5.3.1.3-03
Annex Point IIIA XIII.1.3

Effects on reproduction of birds

Group	Nominal Concentration (mg a.i./kg diet)	Birds Euthanized [a]	Birds Died [b]	Week(s) Found Dead	Initial Number of Birds	Dead (%)	Group Observations
VC	0	9	5	5,7,9,14	40	12.5	Feather loss (head,back), pecking (head), abrasion (head,ear,eye,foot), found dead, and sacrificed.
T1	0.001	5	3	10,11,16	38	7.9	Feather loss (head,back,neck), pecking (head), abrasion (head), hemmorhage (beak). Subdermal hematoma (head), sacrificed, growth on foot, found dead.
T2	0.005	7	2	12,20	40	5.0	Feather loss (head, eye, neck, back), pecking (head), abscess (beak), abrasion (head,foot), sacrificed, subdermal hematoma (head), ataxic, growth on beak, injured (right leg), found dead.
T3	0.020	5	1	7	40	2.5	Feather loss (head,neck), pecking (head), abscess (head), abrasion(foot), hypo-reactivity, abrasion healing, feathers growing, found dead, sacrificed, wing drop, injured (wing), growth on beak, subdermal hematoma (head).
T4	0.100	7	1	16	40	2.5	Feather loss (head, back), pecking (head) abrasion (head,foot), sacrificed, ataxic, fluffed feathers, found dead, growth on beak.

[a] Single birds were euthanized if their pen-mate had died. Both members of a pair were euthanized if the pair was incompatible, described as repeated or routine agonistic behavior which was resulting in severe injury to one or both members of the pair. Both members of some pairs were also euthanized if they met the criterion for excessively low egg production in week 13 of the test. The criterion was egg production in week 13 which was less than or equal to two standard deviations below the mean egg production of the VC group in week 13.
[b] Includes only those birds that were found dead during the 20 week adult observation period. Not included are any birds euthanized during the test.

Section 7.5.3.1.3-03
Annex Point IIIA XIII.1.3

Effects on reproduction of birds

The findings of the terminal necropsies are:

Gross Necropsy Results of Adult *Coturnix* During the Avian Reproduction Test With Difenacoum
(The number in each column represents the number of birds that displayed the listed findings.)

Observations		Nominal Concentrations (mg a.i./kg diet) VC (0)	T1 (0.001)	T2 (0.005)	T3 (0.020)	T4 (0.100)
Fate:	Found dead	5	2	1	1	1
	Sacrificed	35	36	39	39	39
Total necropsies:		40	38	40	40	40
Feather loss:		20	18	19	17	18
Emaciated:		2	1	2	0	1
Breast muscle atrophy:		2	1	2	0	1
Ventriculus contents:	No feed/grit	2	0	0	0	0
	1/2 full	2	4	2	2	2
	Full	36	34	38	38	38
Enlarged: [a]	Liver	n/a	n/a	n/a	n/a	n/a
	Kidneys	0	2	0	0	0
	Spleen	n/a	n/a	n/a	n/a	n/a
	Bile duct	0	0	0	0	0
Discolored:	Liver	6	3	6	1	5
	Heart	0	0	0	0	0
	Kidneys	0	0	1	0	0
	Spleen	0	1	0	0	0
	Bile duct	0	0	0	0	0
Lesions/Abrasions:	Skin	7	5	3	5	4
Lesions/Growths:	Mouth	0	0	0	0	0
	Esophagus/Crop	1	0	0	0	0
	Proventriculus	0	0	0	0	1
	Ventriculus	0	0	0	0	1
	Intestines	0	0	0	0	0
	Heart	0	0	0	0	0
	Liver	0	0	0	0	0
	Bile Duct	0	0	0	0	0
	Spleen	0	0	0	0	0
	Kidneys	0	0	0	0	0
	Uro-Genital	0	0	0	0	0
Reproductive organs:	Mature follicles	18	19	19	20	20
	Egg in oviduct	14	15	15	17	16
	Immature Testes	0	0	1	0	0

[a] Classification as "enlarged" is subjective for kidneys and bile duct. Livers, spleens, and male testes were weighed and the results presented in Table 21 and in Appendix C3.

Organ weights recorded during the terminal necropsies were:

Group	Nominal Concentration (mg a.i./kg diet)	Organ Body Weight (g) Liver [a]	Spleen [b]	Right Testes [c]	Left Testes [d]
		Male			
VC	0	6.0	0.11	3.8	3.7
T1	0.001	7.5	0.16	3.4	3.6
T2	0.005	7.1	0.16	3.2	3.2
T3	0.020	6.7	0.14	3.4	3.6
T4	0.100	6.4	0.18	3.2	3.4
		Female			
VC	0	9.9	0.20	---	---
T1	0.001	10.9	0.27	---	---
T2	0.005	11.6	0.21	---	---
T3	0.020	9.3	0.22	---	---
T4	0.100	10.8	0.21	---	---

[a] Differences in liver weights among males in the groups were not significant when analyzed using ANOVA Male (F = 2.53, calculated F = 2.299). Differences in liver weights among females in the groups were significant when analyzed using ANOVA (F = 2.53, calculated F = 4.693). Although the ANOVA declared significant differences, pair-wise comparisons of each treatment group with the VC group did not find significant differences (Bonferroni's t-test).
[b] Differences in spleen weights among groups were not significant when analyzed using ANOVA Male (F = 2.53, calculated F = 1.693), Female(F = 2.53, calculated F = 0.765).
[c] Differences in right testes weights among groups were not significant when analyzed using ANOVA Male (F = 2.53, calculated F = 1.387).
[d] Differences in left testes weights among groups were not significant when analyzed using ANOVA Male (F = 2.53, calculated F = 0.801).

59.3 Results of controls

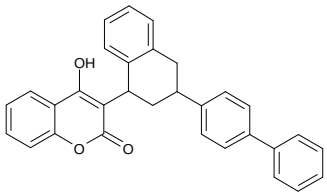
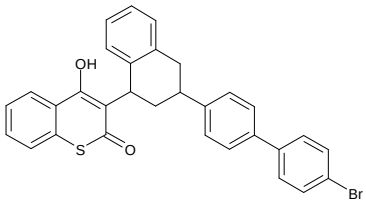
59.3.1 Number/ percentage of animals showing adverse effects

All data for the control group are included in the Tables above.

Section 7.5.3.1.3-03 Annex Point IIIA XIII.1.3	Effects on reproduction of birds	
59.3.2 Nature of adverse effects	Although the listed findings are consistent with anticoagulant exposure, the observations do not form a pattern of consistent effects either within groups or across treatment groups. There were eight cases of sub-lethal observations that could be related to anticoagulant exposure. The six cases were distributed among four groups: VC (n=2), T1 (n=2), T2 (n=2), T3 (n=1), and T4 (n=1) treatment groups. Two control group birds were found to have haemorrhaging in the oesophagus upon necropsy. This illustrates that the birds were incurring many forms of physical stress and tissue damage that was related to aggressive interactions among pen-mates. While some of the sub-lethal conditions observed may be consistent with anticoagulant exposure, similar observations in the control group suggest that there were other causative factors at work. The lack of any systematic dose response in physical symptoms and in any of the other parameters measured in the test support this conclusion.	
	60 APPLICANT'S SUMMARY AND CONCLUSION	
60.1 Materials and methods	Primary Guideline: OECD Test Guideline 206: Avian Reproduction Test, 1984. Secondary Guidelines: Modified in places to follow OECD Test Guideline "Draft Document 1998": Avian Toxicity Test in the Japanese Quail or Japanese Quail, and USEPA Ecological Effects Guideline OPPTS 850.2300: Avian Reproduction Test. No major deviations impacting on study integrity. Treated diet was prepared every two weeks and offered *ad libitum* to groups of 10 male and female pairs for 10 weeks pre-egg laying and 10 weeks egg laying. Treated diets contained nominal 0 (VC), 0.001 (T1), 0.005 (T2), 0.020 (T3) and 0.100 (T4) mg difenacoum/kg diet. Adults were observed daily and diet consumption, body weight, necropsy including wet weights of the liver, spleen and testes recorded. Eggs were collected daily for 10 weeks. The number of eggs laid, eggshell thickness, defective and cracked eggs, viable embryos, live embryos were recorded. Eggs were incubated and hatching success/hatchability, hatchling survival and hatchling body weight at day 14 were recorded. Parameters were analysed statistically.	
60.2 Results and discussion	Of all the parameters measured and analysed in the study, only three were declared to have significant differences, these were: adult female liver weights; the mean body weight of 14-day old hatchlings; and, the mean number of normal hatchlings per hen. The adult female liver weights were significantly different (lower), according to ANOVA, but no significant differences were identified by pair-wise comparisons of each treatment group mean with the VC group. The mean body weight of 14-day old hatchlings found the T3 group to be significantly different (lower) from the VC group but this may have been due to behavioural interactions as the hatchling density in the brooders for this group was the highest. The mean number of normal hatchlings per hen in the T2 and T4 groups was significantly different (lower) from the VC group. These results do not appear as part of a larger pattern. The T2 group contained two pairs having very low numbers of hatchlings. It is therefore considered to be an artifact of the groupings and the analysis process.	

Section 7.5.3.1.3-03 Annex Point IIIA XIII.1.3	Effects on reproduction of birds	
	Regarding the adult generation, although the listed symptoms are consistent with anticoagulant exposure, the observations do not form a pattern of consistent effects either within groups or across treatment groups. There were eight cases of sub-lethal observations that could be related to anticoagulant exposure. Two control group birds were found to have haemorrhaging in the oesophagus upon necropsy. This illustrates that the birds were incurring many forms of physical stress and tissue damage that was related to aggressive interactions among pen-mates. While some of the sub-lethal conditions observed may be consistent with anticoagulant exposure, similar observations in the control group suggest that there were other causative factors at work. The lack of any systematic dose response in physical symptoms and in any of the other parameters measured in the study support this conclusion. Dietary consumption of up to 0.100 mg difenacoum/kg diet had no observed effect on the body weight, feed consumption, or reproductive performance of adult Japanese quail when administered via the diet for 20 weeks. No effects were attributed to the test substance in egg development, or hatchling observations, hatchling body weights and hatchling feed consumption for 14 days. This avian reproduction study was conducted with difenacoum, an AvK anticoagulant rodenticide that serves as a surrogate for difethialone, based on the fact that the two compounds share the same mode of action. They act to form a stable complex with vitamin K. Vitamin K cannot be synthesised by the body. It is involved in the coagulation cascade that leads to blood clotting in response to haemorrhage. Vitamin K is continually recycled in a loop system in the liver, in the activation of clotting factors that are then released into the bloodstream. The loop system employs enzymes, known as vitamin K reductases, to regenerate (recycle) the vitamin K. The anticoagulant rodenticides, also known as antivitamin K or AvK compounds, block the reductase enzymes. The resulting vitamin K/avK/reductase complex is bound to hepatocyte organelle membranes. Ultimately, the finite supply of vitamin K is used up, the production of the activated clotting factor ceases, and the coagulation cascade is interrupted. Once this condition is reached, the organism dies by lethal haemorrhage. Difenacoum is intrinsically less toxic than difethialone, as is indicated by short-term dietary LC_{50} values of 18.9 and 1.9 mg/kg diet respectively, obtained with the same species (mallard) under comparable test conditions. Consequently, the long-term/reproductive NOEC obtained with difenacoum is unlikely to be quantitatively transferable to difethialone. Nevertheless, the following points should be noted: • the Japanese quail were dosed with low-level dietary concentrations of difenacoum for 20 weeks, which far exceeds the duration of any properly-conducted rodenticide baiting campaign; • dietary concentrations of up to and including 0.100 mg difenacoum/kg were tolerated without treatment-related mortalities; • there was no evidence of impaired reproductive performance, hatchling survival or any other adverse sub-lethal effect among any of the groups of birds exposed to difenacoum. This finding is consistent with the argument contained in the waiver	

Section 7.5.3.1.3-03 Annex Point IIIA XIII.1.3	Effects on reproduction of birds	
	presented at Section 7.5.3.1.3-01, *viz*. long-term exposure to sufficiently high dietary concentrations of difethialone will simply result in fatal haemorrhage without any other effects.	
60.2.1 NOEC	The endpoints derived from this study for difenacoum are as follows: **NOEC** (No Observed Effect Concentration): Greater than the T4 dietary concentration: 0.100 mg difenacoum/kg diet administered for 20 weeks. **NOEL** (No Observed Effect Level): Greater than the T4 ingestion level: 0.01138 mg difenacoum/kg body weight/day (mean males and females). Few other avian toxicity endpoints are available for difenacoum, but the short-term dietary LC_{50} obtained with mallard ducks was 18.9 mg difenacoum/kg diet. The corresponding endpoint for difethialone, obtained with mallards under similar conditions, was 1.9 mg difethialone/kg diet. Based on this comparison, a factor of 10 has been applied to derive tentative long-term endpoints for difethialone from those obtained with difenacoum, as follows. **NOEC**: 0.010 mg difethialone; **NOEL**: 0.001138 mg difethialone/kg bw/day. These read-across estimates have been derived from the highest tested dietary concentration in an avian reproduction study with difenacoum in which the effect threshold was not established. The extrapolated NOEC and NOEL values for difethialone should therefore be regarded as being conservative.	x
60.3 Conclusion	The validity criteria can be considered to have been fulfilled. Although the control mortality was very slightly higher than the threshold it is not considered to have affected the integrity of the study. Based on the results of this study with Japanese quail, the NOEC of difenacoum is considered to be greater than 0.100 mg a.i./kg diet. Adult Japanese quail fed difenacoum in the diet for 20 weeks at this level and at three lower levels, did not show any pattern of symptoms consistent with anticoagulant toxicity. There was no suggestion of a dose response at the dietary concentrations listed. All symptoms observed in birds administered difenacoum-treated diets were also observed in the control group. Symptoms observed may have been magnified by the presence of difenacoum in the treated birds, but the degree of interaction cannot be separated and appears to be minor. Difenacoum is structurally closely related to difethialone (see below) and is an antivitamin K (AvK) anticoagulant rodenticide with a similar mode of action. Therefore, this study is considered to be relevant to difethialone in qualitative terms, in that the nature of the toxic effects is expected to be similar for all rodenticides with this mode of action, although the dietary concentrations at which effects are observed may differ.	x

Section 7.5.3.1.3-03 **Annex Point IIIA XIII.1.3**	**Effects on reproduction of birds**	
	difenacoum  Difethialone 	
	Few other avian toxicity endpoints are available for difenacoum, but the short-term dietary LC_{50} obtained with mallard ducks was 18.9 mg difenacoum/kg diet. The corresponding endpoint for difethialone, obtained with mallards under similar conditions, was 1.9 mg difethialone/kg diet. Based on this comparison, a factor of 10 has been applied to derive tentative long-term endpoints for difethialone from those obtained with difenacoum, as follows. **NOEC**: 0.010 mg difethialone; **NOEL**: 0.001138 mg difethialone/kg bw/day. These read-across estimates have been derived from the highest tested dietary concentration in an avian reproduction study with difenacoum in which the effect threshold was not established. The extrapolated NOEC and NOEL values for difethialone should therefore be regarded as being conservative.	
60.3.1 Reliability	1.	x
60.3.2 Deficiencies	None.	
	Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	22.11.2006	
Materials and Methods	See comment under “Remarks”	
Results and discussion	Comment (5.2.1 NOEC): We agree with the applicant’s version that the NOEC for difenacoum is greater than 0.10 mg/kg food. We further agree that a tentative NOEC of < 0.01 mg/kg food can be derived for difethialone.	

Section 7.5.3.1.3-03 Annex Point IIIA XIII.1.3	Effects on reproduction of birds	
Conclusion	Comment (5.3 Conclusion): We agree with the applicant's opinion that the study on difenacoum is relevant with respect to difethialone in qualitative terms, in that the nature of the toxic effects is expected to be similar for all rodenticides with this mode of action. We further agree with the applicant that a factor of 10 can been applied to derive tentative long-term endpoints for difethialone from those obtained with difenacoum. However, it has to be mentioned that there are a few uncertainties regarding this quantitative approach due to interspecies variations with respect to sensitivity and due to a lack of dose-response relationship in most of the tests. Regarding the interspecies variation the following data exists: For the Mallard duck the 5 days LC50 values differ by a factor of about 10 as stated by the applicant. But when comparing the single dose LD50 values for Bobwhite quail (LD50 difethialone = 0.26 mg/kg bw and LD50 difenacoum = 66 mg/kg bw) the difference is much bigger. Furthermore, a Bobwhite quail study is available for difethialone and the 5 days LC50 is 0.56 mg/kg food. For difenacoum two invalid studies are reported for the same species with LC50 values between 0.25 and 7 mg/kg food for the first study and an estimated LC50 of 989 mg/kg food for the other study (LC50 above the highest test concentration). Different species seem to have different sensitivities towards anticoagulant rodenticides but testing of these substances seems to be difficult due to a lack of dose-response relationships and partly high mortalities in control groups. Acute oral tests with birds are not required for anticoagulant rodenticides and therefore the 5 days feeding studies are considered more appropriate for a comparison. The 5 days feeding studies with Bobwhite quail are not considered valid for difenacoum and it seems therefore justified to use the Mallard duck values for difethialone and difenacoum for the derivation of a factor for the sensitivity difference of these two active substances as proposed by the applicant. Moreover, the NOEC for difenacoum is < 0.1 mg/kg and using this value for risk assessment is clearly a worst case. The same applies to a NOEC of 0.01 mg/kg food for difethialone. We agree therefore deriving the following values for difethialone: **NOEC** = 0.010 mg/kg food **NOAEL =** 0.001138 mg/kg bw/day	
Reliability	See comment under "Remarks"	
Acceptability	See comment under "Remarks"	
Remarks	No study evaluation has been done as this avian reproduction study was submitted to the Finnish CA and has already been evaluated by them. Only the parts of the study summary relevant for the read-across from difenacoum to difethialone have been evaluated.	

Table A7.5.3.1.3-11: Method of administration of the test substance

Carrier / Vehicle	Details
Water	No.
Organic carrier	Yes, acetone which was allowed to evaporate.
Concentration of the carrier [% v/v]	2%.
Other vehicle	None.
Function of the carrier / vehicle	Solvent for test substance to facilitate homogeneity.

Table A7.5.3.1.3-12: Test animals (if more than one species is used, for each species one table)

Criteria	Details
Species/strain	*Coturnix coturnix japonica*, strain D1.
Source	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXX
Age (in weeks), sex and initial body weight (bw)	4 weeks old on arrival at test facility, male and female.
Age range within the test	8 weeks old.
Breeding population	Reputable and reliable supplier.
Amount of food	*Ad libitum* feeding.
Age at time of first dosing	8 weeks old.
Health condition / medication	Health condition good, no medication.
Pre-treatment	Acclimatisation period up to 4 weeks, no adverse observations.

Table A7.5.3.1.3-13: Test system

Criteria	Details
Test location	Indoors, in cages.
Holding pens	Test cages used were galvanised steel, 51x50x25.5cm (lxwxh) over a faecal collection pan of absorbent material.
Number of animals (male/female)	198 (99/99).
Number of animals per pen [cm²/bird]	2 animals per cage, surface area 2550cm^2.
Number of animals per dose	40.
Pre-treatment / acclimation	Dry non-medicated Mazuri Exotic Gamebird Starter diet was used. During the last 7days of acclimitisation the diet was changed to Mazuri Exotic Gamebird breeder diet by adding proportionally more breeder diet and less starter diet. Diet and tap water available *ad libitum*.
Diet during test	Mazuri® Exotic Gamebird Breeder was used as the basal feed to prepare all test diets. The test substance

	was dissolved in HPLC-grade acetone to make a stock solution. For each dietary concentration, an appropriate aliquot of the stock solution was transferred to another container and diluted with additional acetone. The total amount of vehicle added to a batch was set at two percent by weight. The final solution for each dietary level was added to the basal feed in the mixing bowl of a large Hobart mixer. Fresh test diets were prepared at least every two weeks. In the early weeks of the study, fresh batches were sometimes mixed more often to assure adequate supplies. Prepared diets were stored in a walk-in freezer for two weeks, at which time a new batch was mixed. No additional supplements were used. Treated diets were offered *ad libitum.*
Dosage levels (of test substance)	Nominal Dietary Concentrations: 0 (VC), 0.001 (T1), 0.005 (T2), 0.020 (T3), 0.100 (T4) mg/kg diet. Treated diets offered *ad libitum* from week 0 to week 20.
Replicate/dosage level	Not applicable.
Dosing method	Dietary.
Dosing volume per application	Diet offered *ad libitum.*
Frequency, duration and method of animal monitoring after dosing	The birds were observed daily during the 20 week exposure period. Birds that died on the test were removed, weighed and necropsied. Feed consumption of each pair of birds was measured weekly during the exposure period. At the conclusion of the treatment period, remaining birds were euthanized and necropsied for gross pathological abnormalities. Specific examination was made on the gastro-intestinal tract, liver, kidneys, bile duct, heart, spleen, and reproductive organs. Wet weights of the liver, spleen and testes were measured at the time of necropsy. Other observations were recorded as necessary.
Time and intervals of body weight determination	The body weight of each bird was measured at the initiation of the 14-day acclimation period, on day 0, at the end of week 8, and at the end of week 20.
Incubation, storing and hatching	All eggs were collected once each day during the 10-week egg laying period. The eggs were placed in an egg cooler (Model ESC-3-110 and ESC-6-110. Kuhl Corporation, Flemington, NJ.) after collection. The egg cooler trays rotate automatically each hour. Temperature and relative humidity of the egg cooler was monitored daily with a digital hygrometer/thermometer. All intact eggs (except eggs used to determine

	eggshell thickness) were set weekly to an incubator (Model 1, Petersime Incubator Company, Gettysburg, OH). The hatchlings were housed in box type poultry brooders (90 cm long x 80 cm wide x 25 cm high). The floor surface area of the brooders was 7200 cm^2. Two brooder compartments were used for each group each week.
Test period after egg-laying	From week 11 to week 20.
Turning of eggs	Yes up to incubation day 15, then placed in a non-turning compartment of the incubator.
Collection period for eggs	From week 11 to week 20.

Table A7.5.3.1.3-14: Test conditions (housing)

Criteria	Details
Test temperature	During the acclimatisation period the mean minimum and maximum daily temperature were 20 and 23ºC, respectively. During the 20 week treatment period the mean minimum and maximum daily temperature were 20 and 23ºC, respectively.
Shielding of the animals	Not stated in the report but the animals were obviously shielded against excessive noise, activity or other disturbance as the study was undertaken as a competent laboratory.
Ventilation	10-15 room changes per hour.
Relative humidity	During the acclimatisation period the mean minimum and maximum relative humidity were 42 and 68% respectively. During the 20 week treatment period the mean minimum and maximum relative humidity were 54 and 73% respectively.
Photoperiod and lighting	Lighting was provided by full spectrum fluorescent bulbs, which were illuminated 7 hours per day during the first eight weeks of the exposure period. At the beginning of the 9th week of the exposure period, the light cycle was increased in increments of two hours per day over 5 days until a light cycle of 17 hours light per day was attained. The average light intensity was 72.5 lux, and was measured at the front of each rack at each level.
Storing, incubation and hatching conditions for eggs	All eggs were collected once each day during the 10-week egg laying period. All intact eggs (except eggs used to determine eggshell thickness) were set weekly to an incubator. On day 15 of incubation, eggs were transferred to the non-turning hatcher compartment that maintained separation of eggs from each parental pen. The hatchlings were removed from the hatcher over a 24-hour period beginning on day 17. Hatchlings were observed for 14 consecutive days after the 24-hour

	hatch period. The hatchlings were housed in box type poultry brooders. Two brooder compartments were used for each group each week. The hatchlings were divided evenly among the two brooders each of the two hatch days, with odd hatchlings (if any) going in one brooder 1 of 2 the first day and brooder 2 of 2 the second day. The most hatchlings occupying a single brooder at any time in this study was 45.
Environmental conditions for young birds	In the hatchling room the mean minimum and maximum temperature were 34 and 37ºC respectively. The mean minimum and maximum relative humidity were 40 and 49% respectively. Lighting was provided by incandescent lighting on a 12 hour cycle.

Table A7.5.3.1.3-15: Values of reproduction ability

Summary of egg data

Group	Nominal Conc. (mg a.i./ kg diet)	Egg Data							
		Eggs Laid	Mean Eggs /Hen /Week	Mean Hatchlings /Hen /Week	Mean Shell Thickness (mm)	Cracked Eggs (%) [a]	Viable Embryo (%) [b]	Live Embryo (%) [c]	Hatch (%) [d]
VC	0	889	6.5	4.0	0.215	9.8	94.4	95.3	83.5
T1	0.001	973	6.4	3.7	0.207	13.4	94.9	96.2	81.9
T2	0.005	963	6.1	3.2	0.216	10.0	87.2	95.2	77.0
T3	0.020	1056	6.2	4.0	0.215	7.7	95.5	96.2	81.4
T4	0.100	1048	6.4	3.8	0.213	9.9	90.7	97.5	83.0

[a] Percent Cracked Eggs = (cracked eggs/eggs candled) * 100.
[b] Percent Viable embryos = (viable embryos/eggs set) * 100.
[c] Percent Live Embryos = (live embryos/viable embryos) * 100.
[d] Percent Hatch = (hatchlings/viable embryos) * 100.

Summary of hatchling data

Group	Nominal Concentration (mg a.i./kg diet)	Hatchling Data				
		Number of Hatchlings	Normal Hatchlings (%) [a]	14-day Normal Survivors/ Normal Hatchlings (%) [b]	14-day Survivors/ Eggs Laid (%) [c]	Mean 14-day Hatchling Body Weight (g)
VC	0	568	96.1	83.9	51.7	68
T1	0.001	565	95.7	88.8	48.9	67
T2	0.005	516	93.2	89.7	45.1	69
T3	0.020	678	94.8	85.1	52.2	59
T4	0.100	629	90.5	88.4	48.4	65

[a] Percent Normal Hatchlings = (normal hatchlings/hatchlings)
[b] Percent 14-day Survivors Per Number of Normal Hatchlings = (14-day normal survivors/normal hatchlings)
[c] Percent 14-day Survivors Per Eggs Laid = (14-day survivors/eggs laid)

Table A7.5.3.1.3-16: Validity criteria for bird reproduction test according to OECD 206

	Fulfilled	Not fulfilled
Mortality of control animals <10%	✓/✗	
Average number of 14-day-old survivors per hen in controls ≥ 14, 12 and 24 for mallard duck, bobwhite quail and Japanese quail	✓	
Average eggshell thickness for the control group ≥ 0.34, 0.19 and 0.19 mm for mallard duck, bobwhite quail and Japanese quail	✓	
Concentration of the test substance in the diet ≥ 80 % of the nominal concentration throughout the test period	✓	

Mortality of the control animals was 12.5%, the highest of any of the groups.

Section A7.5.5.1-01 **Annex Point IIIA XIII.1.3**	**Bioconcentration (terrestrial), further studies**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [x]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [x]**	
Detailed justification:	The Directive 98/8/EC states in Article 8 (5) that "*information which is not necessary owing to the nature of the biocidal product or of its proposed uses need not be supplied. The same applies where it is not scientifically necessary or technically possible to supply the information. In such cases, a justification, acceptable to the competent authority must be submitted…*".	
	Exposure According to the 'Emission scenario document for biocides used as rodenticides' (EUBEES 2), exposure of the soil compartment to active substances contained in rodenticidal baits is feasible, with concentrations dependent on deployment practices and product type. Exposure of the terrestrial compartment will be extremely localised and confined to small concentration 'hotspots' within a few cm of bait points located outdoors. The scope for widespread and significant exposure of the terrestrial compartment is therefore negligible.	
	Bioaccumulation in soil organisms Those substances with a log K_{ow} of > 3 may bioaccumulate in biota. However, other processes such as a high propensity to adsorb to soil, hydrolysis or other soil dissipation processes may reduce or eliminate bioaccumulation. Therefore, the use of log K_{ow} by itself is a poor indicator of bioaccumulation. There are not currently available guidance procedures to reliably estimate the potential for biocide substances to bioaccumulate in soil organisms. However, for plant protection products, the EU Commission guidance document SANCO 4145/2000 (25 September 2002) does provide guidance on the estimate of bioaccumulation and food chain behaviour in relation to risks to non-target vertebrates. In SANCO/4145/2000 a simple, worst-case, assessment to estimate the bioconcentration factor from soil to earthworm is given as follows: $BCF = (0.84 + 0.01\ K_{ow}) / f_{oc}.K_{oc}$ - where f_{oc} is the organic content of soil and has a default value of 0.02 and K_{oc} is the organic carbon adsorption coefficient. For difethialone, the highest log K_{ow} is 6.29 (Section A.3). The highest K_{ow} is thus 1,949,845 and the Koc is $\geq 1.0 \times 10^8$ mL/g (Doc. II-A, Section 4.1.2). Based on these parameters the estimated BCF (concentration in soil relative to the concentration in an earthworm) is 9.7×10^{-3}. Therefore, the propensity for difethialone to bioaccumulate in terrestrial soil dwelling organisms is very low, mainly due to its high soil adsorptive properties.	X X

Section A7.5.5.1-01 Annex Point IIIA XIII.1.3	Bioconcentration (terrestrial), further studies	
	Consequences for risks to higher trophic levels As a result of the very low bioconcentration potential of difethialone in the soil compartment and the negligible soil exposure following the use of products containing difethialone, higher trophic levels are unlikely to be at risk. The risks to higher trophic levels are, therefore, confined to an assesment of exposure via ingestion of pellets and secondary exposure via ingestion of target rodents (Doc. II). **Conclusion** Based on the negligible exposure and very low potential to bioaccumulate in the soil compartment further studies of the bioconcentration of difethialone in the terrestrial compartment are consequently unnecessary.	x
Undertaking of intended data submission [ ]	Not applicable.	
	Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted	
	EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	6 January 2005, revised 28 December 2006	
Evaluation of applicant's justification	Agree with applicant's version **Comment:** The applicant has performed a theoretic estimation of the bioconcentration factor from soil to earthworm by use of an empirical derived equation ($BCF=9.7 \times 10^{-3}$). According to TGD the equation suggested for worms in water is: BCF=(0.84+0.012Kow)/RHOearthworm, using Log Kow=6.3 and RHO=1 give BCF=23,943 l/kg. Difethialone strongly adsorbs to organic matter. However, earthworms also able to take up chemicals from food. According to TGD the uptake of chemicals via food may affect accumulation at a log Kow > 5. The BCF of 23,943 l/kg is used for a secondary poisoning assessment via the terrestrial food chain. The potential of bioaccumulation of difethialone in earthworm is expected to be high. However, no further testing is considered necessary because exposure to soil is limited in space 10 cm around a bait station.	
Conclusion	Acceptable	
Remarks		