Section A7.4.1.1 Acute toxicity to fish

Annex Point IIA VII.7.1

			Official use only
		1 REFERENCE	
1.1	**Reference**	Report: The Toxicity to Rainbow Trout (*Oncorhynchus mykiss*) of DIFENACOUM Technical. XXXXX - March 2003. XXXXX report ENV5794/120139 Acute toxicity study of test substance difenacoum technical.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with Access to data	PelGar International Ltd. Activa srl	
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	
1.1	**Guideline study**	OECD 203	
1.2	**GLP**	Yes	
1.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
1.1	**Test material**	As given in section 2	
1.1.1	Lot/Batch number	ECO120139	
1.1.2	Specification	As given in section 2	
1.1.3	Purity	99.7 % difenacoum	x
1.1.4	Composition of Product	Not applicable	
1.1.5	Further relevant properties	Not Applicable	
1.1.6	Method of analysis	HPLC	x
1.2	**Preparation of TS solution for poorly soluble or volatile test substances**	See table A7_4_1_1-1)	x
1.3	**Reference substance**	Yes	x
1.3.1	Method of analysis for reference substance	Acute toxicity: 96 hour LC_{50} of Potassium dichromate on rainbow trout with test concentrations of, 0 (control), 32, 56, 100, 180 and 320mg/l.	x
1.4	**Testing procedure**		
1.4.1	Dilution water	See table A7_4_1_1-2	x
1.4.2	Test organisms	See table A7_4_1_1-3	x
1.4.3	Test system	See table A7_4_1_1-4	x
1.4.4	Test conditions	See table A7_4_1_1-5	x
1.4.5	Duration of the test	96 hours	

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1.4.6	Test parameter	Mortality	
1.4.7	Sampling	Test substance analysis of each concentration carried out as soon as possible after sampling at 0, 24, 48, 72 and 96 hours with samples being frozen until analysis	
1.4.8	Monitoring of TS concentration	Yes Start of study, before and after renewal of solutions at 24, 48, 72 hours, and at the end of the 96 hour exposure period.	
1.4.9	Statistics	LC_{50} determined by the Spearman-Karber method NOEC and LOEC determined by Fisher's Exact test	

4 RESULTS

1.1	**Limit Test**	Not performed	
1.1.1	Concentration	Not Applicable	
1.1.2	Number/ percentage of animals showing adverse effects	Not Applicable	
1.1.3	Nature of adverse effects	Not Applicable	
1.2	**Results test substance**		
1.2.1	Initial concentrations of test substance	0 (control), 0.13, 0.25, 0.5, 1.0 and 2.0 mg/l	x
1.2.2	Actual concentrations of test substance	see table below	x
1.2.3	Effect data (Mortality)	Mortality: see table A7_4_1_1-6 LC_{50} plus 95% confidence limits: see table A7_4_1_1-7	
1.2.4	Concentration / response curve	see table below	x

mg/l Nom	**0**	**0.13**	**0.25**	**0.5**	**1.0**	**2.0**
0 hrs fresh	0	0.12	0.24	0.38	0.90	1.94
24 hrs aged	0	0.11	0.21	0.43	0.83	1.54
24 hrs renewed	0	0.12	0.22	0.42	*	-
48 hrs aged	0	0.10	0.21	0.41	0.83	-
48 hrs renewed	0	0.11	0.22	0.46	-	-
72 hrs aged	0	0.10	0.18	0.40	-	-
72 hrs renewed	0	0.12	0.22	0.53	-	-

* Extraction error, sample not available for analysis

\- No replacement solutions required

Exposure period (hours)	**Nominal concentration (mg/l) Control**	**0.13**	**0.25**	**0.50**	**1.0**	**2.0**
0	0	0	0	0	0	0
24	0	0	0	0	28.6	100
48	0	0	0	28.6	100	100
72	0	0	0	71.4	100	100

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		96	0	0	0	85.7	100	100

1.2.5	Other effects	Not stated
1.3	**Results of controls**	
1.3.1	Number/ percentage of animals showing adverse effects	No effects
1.3.2	Nature of adverse effects	No effects
1.4	**Test with reference substance**	Performed
1.4.1	Concentrations	Potassium dichromate at nominal concentrations 0(control), 32,56, 100, 180 and 320 mg/l
1.4.2	Results	24 hour LC_{50} = 240 mg/l (206 – 279 mg/l; 95% confidence limits) 48 hour LC_{50} = 240 mg/l (206 – 279 mg/l; 95% confidence limits) 72 hour LC_{50} = 133 mg/l (91 – 201 mg/l; 95% confidence limits) 96 hour LC_{50} = 133 mg/l (91 – 201 mg/l; 95% confidence limits)

5 APPLICANT'S SUMMARY AND CONCLUSION

1.1	**Materials and methods**	OECD 203
1.2	**Results and discussion**	Test substance is extremely insoluble in water(c.0.03ppm), of very low vapour pressure, and is subject to rapid photolysis (half-life c.7.5 hrs)
1.2.1	LC_0	48 hour LC_0 = 0.50mg/l (as determined by Fisher's Exact test) 96 hour LC_0 = 0.25mg/l (as determined by Fisher's Exact test)
1.2.2	LC_{50}	24 hour LC_{50} = 1.2 mg/l (0.92 – 1.5 mg/l; 95% confidence limits) 48 hour LC_{50} = 0.58 mg/l (0.46 – 0.74 mg/l; 95% confidence limits) 72 hour LC_{50} = 0.43 mg/l (0.34 – 0.55 mg/l; 95% confidence limits) 96 hour LC_{50} = 0.39 mg/l (0.33 – 0.47 mg/l; 95% confidence limits)
1.2.3	LC_{100}	48 hour LC_{100} = 1.0mg/l 96 hour LC_{100} = 1.0mg/l
1.3	**Conclusion**	R50 /R53 Very toxic to aquatic organisms applies. All validity criteria can be considered as fulfilled. Clear dose-response relationship shown (see table A7_4_1_1-8)
1.3.1	Other Conclusions	
1.3.2	Reliability	1
1.3.3	Deficiencies	No

Evaluation by Competent Authorities

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	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	22.6.2006
Materials and Methods	3.1.3: Purity of test substance not mentioned in the test report. 3.1.6: Limit of quantification not given in the test report. 3.3: Test of reference substance given in Appendix 2, but not mentioned elsewhere in the test report. Test with reference substance conducted earlier in 2001. 3.3.1: Reference substance not analysed. 3.4.3: Semi-static test. Table A7_4_1_1:1: Concentration of the solvent DMF not mentioned (concentration should not exceed 100 mg/l). Table A7_4_1_1:2 In the table values for alkalinity, pH dissolved oxygen and conductance of dilution water are given, but no values have been given for these parameters in the test report. Table A7_4_1_1:3: Mean length and weight of fish given in the table refer to length and weight at the end of the test. Kind of food, amounf food and feeding frequency are given in the table, but not given in the test report. Table A7_4_1_1:4. Volume/animal reported in the table (10 L/7 fish), but not given in the test report. The test vessels contained probably less test medium than 10 l which was the volume of the test vessels. In the table it is mentioned that test vessels were not closed,. but this fact was not given in the test report. Table A7_4_1_1:5: In the table it is mentioned that dilution water was not aerated or pH was not adjusted, but nothing is mentioned about aeration of adjustment of pH in the test report.
Results and discussion	4.2.1-4.2.2-4.2.4: Lowest nominal test concentration 0.06 mg/l, this concentration was not verified analytically. The reason for not analysing this concentration was not given. In the test report p. 7 the nominal concentrations of 0.06, 0.13, 0.25, 0.5 and 1.0 mg/l given. In Appendices 1 and 3 the highest test concentration of 2.0 mg/l is given. The validity criteria were fulfilled, except that measured concentrations of aged solutions dropped a little below 80% (73-85%). Photolytic degradation may have contributed to the declining concentrations. 96 hour LC_{50} = 0.33 mg/l (0.28 – 0.4 mg/l; 95% confidence limits) based on the measured concentration concentrations (The results recalculated on the basis of measured concentration are shown after Table A7_4_1_1-8). The results showed a dose-response relationship.
Conclusion	Difenacoum is very toxic to fish.
Reliability	2
Acceptability	Acceptable.
Remarks	The test concentrations were below 80% of nominals (fresh solutions: 77-105%, aged solutions: 73-85%) and hence the test results should have been based on the measured concentrations. The loading of fish exceeded the maximum recommended in the OECD 203 (1 g fish/litre), but is not assumed to affect the test result.
	COMMENTS FROM ...

Section A7.4.1.1 Acute toxicity to fish

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Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A7_4_1_1-1: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Dispersion	Yes
Vehicle	DMF
Concentration of vehicle	Not stated
Vehicle control performed	Yes
Other procedures	None

Table A7_4_1_1-2: Dilution water

Criteria	Details
Source	De-chlorinated mains tap water
Alkalinity	214 mg/l
Hardness	286 mg/l calcium carbonate
pH	6.6 – 8.5
Oxygen content	99-101% ASV
Conductance	766µS/cm
Holding water different from dilution water	No

Table A7_4_1_1-3: Test organisms

Criteria	Details
Species/strain	Rainbow Trout (*Oncorhynchus mykiss*)
Source	Bilbury Trout Farm, Cirencester
Wild caught	No
Age/size	Mean length: 48.4 mm Weight: 1.47 g
Kind of food	Keystart Fingerling 25
Amount of food	Feed at 1% of body weight per day
Feeding frequency	Daily
Pretreatment	No
Feeding of animals during test	No

Table A7_4_1_1-4: Test system

Criteria	Details
Test type	Semi-static
Renewal of test solution	Test solutions replaced every 24 hours
Volume of test vessels	10 litre volume plastic aquaria
Volume/animal	1.43 litres/fish
Number of animals/vessel	7
Number of vessels/ concentration	7 vessels: 0 (control), DMF (control), 0.13, 0.25, 0.5,

	1.0 and 2.0 mg/l
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_1_1-5: Test conditions

Criteria	Details
Test temperature	13.0 – 15.0°C
Dissolved oxygen	94 – 101%
PH	7.4 – 8.4
Adjustment of pH	No
Aeration of dilution water	No
Intensity of irradiation	
Photoperiod	16 hours light and 8 hours dark

Table A7_4_1_1-6: Mortality data

Test-Substance Concentration (nominal) [mg/l]	Mortality							
	Total Number				Cumulative Percentage			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
0 (Control DMF)	0	0	0	0	0	0	0	0
0.13	0	0	0	0	0	0	0	0
0.25	0	0	0	0	0	0	0	0
0.50	0	2	5	6	0	28.6	71.4	85.7
1.0	2	7	7	7	28.6	100	100	100
2.0	7	7	7	7	100	100	100	100
Temperature [°C]	13	14.5	14.5	14.0				
pH	8.4	8.3	8.3	8.4				
Oxygen [mg/l]	99-101	94-100	96-100	99-100				

Table A7_4_1_1-7: Effect data

	48 h [mg/l][1]	95 % c.l.	96 h [mg/l][1]	95 % c.l.
LC_0	-	-	-	-
LC_{50}	0.58(n)	0.46 – 0.74	0.39(n)	0.33 – 0.47
LC_{100}	-	-	-	-

[1] indicate if effect data are based on nominal (n) or measured (m) concentrations

Table A7_4_1_1-8: 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	**YES**	
Criteria for poorly soluble test substances	**YES**	

Substance under test: **DIFENACOUM Technical**

Chemex reference: **Sample: ECO120139**
Study: ENV5794

Test species: **Rainbow Trout (*Oncorhynchus mykiss*)**

Nominal conc.	**0.13**	**0.25**	**0.5**	**1.0**	**2.0**
	Analysed Measured concentration				
0 hrs fresh	0.12	0.24	0.38	0.90	1.94
24 hrs aged	0.11	0.21	0.43	0.83	1.54
24 hrs fresh	0.12	0.22	0.42		
48 hrs aged	0.10	0.21	0.41	0.83	
48 hrs fresh	0.11	0.22	0.46		
72 hrs aged	0.10	0.18	0.40		
72 hrs fresh	0.12	0.22	0.53		
Mean measured	**0.11**	**0.21**	**0.43**	**0.85**	**1.74**
concentration					
% recovery of nominal	86	86	87	85	87

CUMULATIVE PERCENT MORTALITIES

Exposure period (hours)	**Mean measured concentration (mg/l)**					
	Control DMF	**0.11**	**0.21**	**0.43**	**0.85**	**1.74**
0	0	0	0	0	0	0
24	0	0	0	0	28.6	100
48	0	0	0	28.6	100	100
72	0	0	0	71.4	100	100
96	0	0	0	85.7	100	100

LC_{50} value Mean Measured concentration

Period of exposure		**LC_{50} value (mg/l) with 95% Confidence limits**
Hours	**Days**	
96	4	0.33mg/l (0.28 – 0.40) by Spearman Karber method

Section A7.4.1.2 Acute toxicity to invertebrates

Annex Point IIA VII.7.2 Acute toxicity to *Daphnia magna*

			Official use only
		1 REFERENCE	
1.4	**Reference**	Report: The Toxicity to *Daphnia magna* of DIFENACOUM Technical. XXXXX - March 2003. XXXXX report - ENV5793/120139 Acute toxicity study of test substance difenacoum technical.	
1.5	**Data protection**	Yes	
1.5.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.5.2	Companies with Access to data	PelGar International Ltd. Activa srl	
1.5.3	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**	OECD 202	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.2	Lot/Batch number	ECO120139	
3.1.3	Specification	As given in section 2	
3.1.4	Purity	99.7 % difenacoum	x
3.1.5	Composition of Product	Not applicable	
3.1.6	Further relevant properties	Not Applicable	
3.1.7	Method of analysis	HPLC	x
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	See table A7_4_1_2-1	
3.3	**Reference substance**	Yes	x
3.3.2	Method of analysis for reference substance	Aquatic toxicity: 48 hour EC_{50} of potassium dichromate on *Daphnia magna*. Concentrations: 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg/	x
3.4	**Testing procedure**		
3.4.1	Dilution water	See table A7_4_1_2-2	x
3.4.2	Test organisms	See table A7_4_1_2-3	
3.4.3	Test system	See table A7_4_1_2-4	x
3.4.4	Test conditions	See table A7_4_1_2-5	x
3.4.5	Duration of the test	48 hours	

Section A7.4.1.2 Acute toxicity to invertebrates

Annex Point IIA VII.7.2 Acute toxicity to *Daphnia magna*

3.4.6	Test parameter	Immobility
3.4.7	Sampling	Test substance analysis of each concentration carried out as soon as possible after sampling at 0, 24 and 48 hours with samples being frozen until analysis
3.4.8	Monitoring of TS concentration	Yes Start of study, before and after renewal of solutions at 24 hours, and at the end of the 48 hour exposure period.
3.4.9	Statistics	EC_{50} determined by the Maximum Likelihood-Probit method NOEC and LOEC determined by Fisher's Exact test
		4 RESULTS
4.4	**Limit Test**	Not performed
4.4.1	Concentration	Not Applicable
4.4.2	Number/ percentage of animals showing adverse effects	Not Applicable
4.4.3	Nature of adverse effects	Not Applicable
4.5	**Results test substance**	
4.5.1	Initial concentrations of test substance	0 (control), 0.13, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/l

4.5.2 Actual concentrations of test substance

mg/l Nom	**0**	**0.13**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**
0 hrs fresh	0	0.10	0.16	0.39	0.78	1.45	3.34
24 hrs aged	0	0.07	0.15	0.27	0.58	1.16	3.28
24 hrs renewed	0	0.09	0.14	0.40	0.98	1.77	3.37
48 hrs aged	0	0.09	0.18	0.38	0.66	1.63	3.27

4.5.3	Effect data (Immobilisation)	Immobility: See table A7_4_1_2-6; EC_{50} and 95% confidence limits: See table A7_4_1_2-7
4.5.4	Concentration / response curve	Slope: 2.9 with 95% confidence limits 2.0 – 3.8.
4.5.5	Other effects	None stated
4.6	**Results of controls**	No effects
4.7	**Test with reference substance**	Performed
4.7.1	Concentrations	Potassium dichromate at nominal concentrations 0 (control), 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg /l
4.7.2	Results	24 hour EC_{50} = 1.3 mg/l (1.1 – 1.6 mg/l; 95% confidence limits) 48 hour EC_{50} = 0.9 mg/l (0.8 – 1.1 mg/l; 95% confidence limits)

Section A7.4.1.2 Acute toxicity to invertebrates

Annex Point IIA VII.7.2 Acute toxicity to *Daphnia magna*

		48 hour NOEC = 0.56 mg/l 48 hour 100% mortality = 3.2 mg/l
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.4	**Materials and methods**	OECD 202
5.5	**Results and discussion**	Test substance is extremely insoluble in water (c.0.03ppm), of very low vapour pressure, and is subject to rapid photolysis (half-life c.7.5 hrs)
5.5.1	EC_0	-
5.5.2	EC_{50}	24 hour EC_{50} = >4.0 mg/l (not possible to determine 95% confidence limits) 48 hour EC_{50} = 1.2 mg/l (0.95 – 1.6 mg/l; 95% confidence limits)
5.5.3	EC_{100}	-
5.6	**Conclusion**	R51 /R53 Toxic to aquatic organisms applies. All validity criteria can be considered as fulfilled. Clear dose-response relationship shown (see table A7_4_1_2-8)
5.6.1	Reliability	1
5.6.2	Deficiencies	No

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	22.6.2006
Materials and Methods	3.1.3: Purity of difenacoum not mentioned in the test report. 3.1.6: Limit of quantification not given in the test report. 3.4.3: Semi-static test. Table A7_4_1_2-2: Alkalinity, Ca / Mg ratio, Na /K ratio and conductance given in the table, but not reported in the test report. Table A7_4_1_2-4: Test vessels were covered with a transparent perpex sheet. Table A7_4_1_2-5: Dilution water was aerated obviosuly before the start of the test.
Results and discussion	48 hour EC_{50} = 0.91 mg/l (0.70 – 1.21 mg/l; 95% confidence limits) based on the measured concentration (Recalculated results based on the measured concentrations are given after Table A7_4_1_2-8). Measured concentrations were 54-98% of nominal concentrations. The results showed a dose-response relationship. Half of the test concentrations and the EC_{50} exceeded water solubility of difenacoum at pH 7 (0.5 mg/l). However, during the test pH was 7.7-8.0 and hence a higher water solubility is assumed in the test conditions. The validity criteria were fulfilled.
Conclusion	Difenacoum is very toxic to *Daphnia magna*.

Section A7.4.1.2 Acute toxicity to invertebrates

Annex Point IIA VII.7.2 Acute toxicity to *Daphnia magna*

Reliability	2
Acceptability	Acceptable
Remarks	The test concentrations were below 80% of nominals (fresh solutions: 57-98%, aged solutions: 54-82%) and hence the test results should have been based on the measured concentrations. Photolytic degradation may have contributed to the declining concentrations.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A7_4_1_2-1: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Dispersion	Yes
Vehicle	DMF
Concentration of vehicle	Not stated
Vehicle control performed	Yes
Other procedures	None

Table A7_4_1_2-2: Dilution water

Criteria	Details
Source	De-chlorinated mains tap water
Alkalinity	214 mg/l
Hardness	240 mg/l $CaCO_3$
pH	7.0 – 8.0
Ca / Mg ratio	18.6 : 1
Na / K ratio	5.2 : 1
Oxygen content	Minimum of 60% air saturation
Conductance	766µS/cm
Holding water different from dilution water	No

Table A7_4_1_2-3: Test organisms

Criteria	Details
Strain	*Daphnia magna*
Source	Shell Research Laboratories
Age	Less than 24 hours
Breeding method	Normal
Kind of food	A suspension of *Chlorella vulgaris*
Amount of food	1 mg organic carbon per litre of culture water
Feeding frequency	Daily
Pretreatment	None
Feeding of animals during test	No

Table A7_4_1_2-4: Test system

Criteria	Details
Renewal of test solution	Renewed after 24 hours
Volume of test vessels	25 ml of solution in 50 ml vessel
Volume/animal	5 ml
Number of animals/vessel	5
Number of vessels/ concentration	4
Test performed in closed vessels due to significant	No

volatility of TS	

Table A7_4_1_2-5: Test conditions

Criteria	Details
Test temperature	20 ± 1ºC
Dissolved oxygen	0hr = 96% ASV; 24hr (before renewal) = 100% ASV 24hr (after renewal) = 98% ASV; 48hr = 100% ASV
pH	0hr = 7.8 – 8.0; 24hr (before renewal) = 7.7; 24hr (after renewal) = 7.9; 48hr = 7.8 –7.9.
Adjustment of pH	No
Aeration of dilution water	No
Quality/Intensity of irradiation	Light intensity = 350 lux
Photoperiod	16 hours light and 8 hours dark

Table A7_4_1_2-6: Immobilisation data

Test-Substance Concentration (nominal/effective) [1] [mg/l]	Immobile *Daphnia*						
	Number		Percentage		Oxygen [%ASV]	pH	Tempera-ture [°C]
	24 h	48 h	24 h	48 h	48 h	48 h	48 h
0	0	0	0	0	100	7.8	20
|DMF control	0	0	0	0	100	7.9	20
0.13	0	0	0	0	100	7.9	20
0.25	0	1	0	5	100	7.9	20
0.50	0	2	0	10	100	7.9	20
1.0	0	9	0	45	100	7.9	20
2.0	2	12	10	60	100	7.9	20
4.0	4	20	20	100	100	7.9	20

[1] specify, if TS concentrations were nominal or measured

Table A7_4_1_2-7: Effect data

	EC_{50}[1]	95 % c.l.	EC_0[1]	EC_{100}[1]
24 h [mg/l]	>4.0 (n)	Not possible to determine	1.0	>4.0
48 h [mg/l]	1.2 (n)	0.95 – 1.6	0.13	4.0

[1] indicate if effect data are based on nominal (n) or measured (m) concentrations

Table A7_4_1_2-8: Validity criteria for acute daphnia immobilisation test according to OECD Guideline 202

	fulfilled	Not fulfilled
Immobilisation of control animals <10%	**Yes**	
Control animals not staying at the surface	**Yes**	
Concentration of dissolved oxygen in all test vessels >3 mg/l	**Yes**	
Concentration of test substance ≥80% of initial concentration during test	**Yes**	
Criteria for poorly soluble test substances ergänzen	**Yes**	

Substance under test: **DIFENACOUM Technical**

Chemex reference: **Sample: ECO120139**
Study: ENV5793

Test species: ***Daphnia magna***

Nominal conc. mg/l	**0.13**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**
	Analysed measured concentration					
0 hrs fresh	0.1	0.16	0.39	0.78	1.45	3.34
24 hrs aged	0.07	0.15	0.27	0.58	1.16	3.28
24 hrs fresh	0.09	0.14	0.4	0.98	1.77	3.37
48 hrs aged	0.09	0.18	0.38	0.66	1.63	3.27
Mean	**0.09**	**0.16**	**0.36**	**0.75**	**1.50**	**3.32**
Measured conc.						
% recovery of Nominal concentration	67	63	72	75	75	83

Cumulative percent immobilisation

Concentration Mean measured (mg/l)	**Number immobilised**		**% immobilisation**	
	24 hours	**48 hours**	**24 hours**	**48 hours**
0	0	0	0	0
DMF control	0	0	0	0
0.09	0	0	0	0
0.16	0	1	0	5
0.36	0	2	0	10
0.75	0	9	0	45
1.5	2	12	10	60
3.32	4	20	20	100

EC_{50} values Mean Measured concentration

Period of exposure (hours)	**EC_{50} value (mg/l)**	**95% confidence limits (mg/l)**
24	>3.32	Not possible to determine
48	0.91	0.70 – 1.21

Section A7.4.1.3 Growth inhibition test on algae

Annex Point IIA VII.7.3

			Official use only
		1 REFERENCE	
1.1	**Reference**	Report: The Growth Inhibition of the alga *Selenastrum capricornutum* by DIFENACOUM Technical. XXXXX - March 2003. XXXXX. Report -ENV5792/120139	
		Toxicity study of test substance difenacoum technical.	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with Access to data	PelGar International Ltd. Activa srl	
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**	OECD 201	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	ECO120139	
3.1.2	Specification	As given in section 2	
3.1.3	Purity	99.7 % difenacoum	x
3.1.4	Composition of Product	Not applicable	
3.1.5	Further relevant properties	Not Applicable	
3.1.6	Method of analysis	HPLC	x
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	See table A7_4_1_3-1)	
3.3	**Reference substance**	Yes	x
3.3.1	Method of analysis for reference substance	Aquatic toxicity: 72 hour EC_{50} of Potassium dichromate on *Selenastrum capricornutum* with test concentrations of, 0 (control), 0.18, 0.32, 0.56, 1.0 and 1.8 mg/l.	x
3.4	**Testing procedure**		
3.4.1	Culture medium	***Nutrient Final concentration in culture medium (mg/l)*** NH_4Cl 15 $MgCl_2.6H_2O$ 12 $CaCl_2.2H_2O$ 18 $MgSO_4.7H_2O$ 15 KH_2PO_4 1.6	

Section A7.4.1.3 Growth inhibition test on algae

Annex Point IIA VII.7.3

		$FeCl_3 .6H_2O$ 0.08
		$Na_2EDTA.2H_2O$ 0.1
		H_3BO_3 0.185
		$MnCl_2.4H_2O$ 0.415
		$ZnCl_2$ 0.003
		$CoCl_2.6H_2O$ 0.0015
		$CuCl_2.2H_2O$ 0.00001
		$Na_2MoO_4.2H_2O$ 0.007
		$NaHCO_3$ 50
3.4.2	Test organisms	see table A7_4_1_3-2
3.4.3	Test system	see table A7_4_1_3-3
3.4.4	Test conditions	see table A7_4_1_3-4
3.4.5	Duration of the test	72 hours
3.4.6	Test parameter	Cell multiplication inhibition
3.4.7	Sampling	Sampling at 0, 24, 48 and 72 hrs
3.4.8	Monitoring of TS concentration	Yes Start and end of test period
3.4.9	Statistics	EC_{50} values logarithm-linear or logarithm-probit plot
		4 RESULTS
4.1 Limit Test		Not performed
4.1.1	Concentration	Not applicable
4.1.2	Number/ percentage of animals showing adverse effects	Not applicable
4.2 Results test substance		
4.2.1	Initial concentrations of test substance	0 (control), DMF, 0.10, 0.18, 0.32, 0.56 and 1.0 mg/l

4.2.2 Actual concentrations of test substance

mg/l Nom	**0**	**0.10**	**0.18**	**0.32**	**0.56**	**1.0**
0 hrs fresh	0	0.09	0.14	0.27	0.49	0.82
72 hrs aged	0	0.07	0.12	0.19	0.37	0.63

4.2.3 Growth curves

4.2.4 Concentration / response curve

Concentration (mg/l)	**Cell density measurements (cells/ml x 10^4)** **24 hours**	**48 hours**	**72 hours**
0	4.50	26.33	114.89
0.10	4.33	18.22	71.11

Section A7.4.1.3 Growth inhibition test on algae

Annex Point IIA VII.7.3

		0.18 4.33 17.89 61.11	
		0.32 3.11 9.89 29.22	
		0.56 3.56 7.44 20.33	
		1.0 1.89 3.89 4.89	
4.2.5	Cell concentration data	see table A7_4_1_3-5	
4.2.6	Effect data (cell multiplication inhibition)	E_bC_{50} 0 - 72 hrs 0.19 mg/l E_rC_{50} 0 - 72 hrs 0.68 mg/l NOE_rC for 0 - 72 hrs 0.18 mg/l	x
4.2.7	Other observed effects	None stated	
4.3	**Results of controls**	No effects	
4.4	**Test with reference substance**	Performed	
4.4.1	Concentrations	Potassium dichromate at nominal concentrations 018, 0.32, 0.56, 1.0 and 1.8 mg/l	x
4.4.2	Results	E_bC_{50} 0 – 48 hrs 0.59 mg/l E_rC_{50} 0 - 48 hrs 0.86 mg/l E_bC_{50} 0 - 72 hrs 0.58 mg/l E_rC_{50} 0 - 72 hrs 0.88 mg/l	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	OECD 201	
5.2	**Results and discussion**	Test substance is extremely insoluble in water (c.0.03ppm), of very low vapour pressure, and is subject to rapid photolysis (half-life c.7.5 hrs)	
5.2.1	NOE_rC	0 – 48 hrs NOE_rC = 0.32 mg/l	x
5.2.2	E_rC_{50}	0 – 48 hrs = 0.86 mg/l; 0 – 72 hrs = 0.88 mg/l	
5.2.3	E_bC_{50}	0 – 48 hrs = 0.59 mg/l; 0 – 72 hrs = 0.58 mg/l	
5.3	**Conclusion**	R50 /R53 Very toxic to aquatic organisms applies. All validity criteria can be considered as fulfilled. Clear dose-response relationship shown	
5.3.1	Reliability	1	
5.3.2	Deficiencies	No	

Section A7.4.1.3 Growth inhibition test on algae

Annex Point IIA VII.7.3

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	22.6.2006
Materials and Methods	3.1.3: Purity of difenacoum not given in the test report. 3.1.6: Limit of quantification not reported. 3.3 Reference substance mentioned only in Appendix 3. 3.3.1: Analysis of reference substance not mentioned. Table A7_4_1_3-3: 3 test vessels per treatment. In the table it is stated that the test was not conducted in closed vessels, but this fact is not reported in the test report.
Results and discussion	4.2.6: NOEC not given in the test report. 4.4.1: The lowest reference substance concentration is 0.18 mg/l. 72 hrs E_rC_{50} = 0.14 mg/l based on the measured concentrations (The recalculated results based on the measured concentrations are given after Table A7_4_1_3-6). The validity criteria were fulfilled.
Conclusion	Difenacoum is very toxic to algae.
Reliability	2
Acceptability	Acceptable.
Remarks	The test concentrations were below 80% of nominals (fresh solutions: 79-91%, 72 hrs aged solutions: 59-67%). Photolytic degradation may have contributed to the declining concentrations The RMS cannot follow the calculation of percentage inhibition by growth rate after 72 hour (Test report, p. 9). The percentage inhibitions calculated on the basis of mean cell density measurements according to the OECD 201 are: Control: 0%, 0.1 mg/l: 14%, 0.18 mg/l: 18%, 0.32 mg/l: 31%, 0.56 mg/l: 46%, and 1.0 mg/l: 71%. The percentage inhibitions after 48 hours were not checked.
	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A7_4_1_3-1: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Dispersion	Yes
Vehicle	DMF
Concentration of vehicle	Not stated
Vehicle control performed	Yes
Other procedures	None

Table A7_4_1_3-2: Test organisms

Criteria	Details
Species	*Selenastrum capricornutum*
Strain	CCAP278/4
Source	Culture Collection of Algae and Protoza Institute of Freshwater Ecology Windermere Laboratory
Laboratory culture	Yes
Method of cultivation	Not stated
Pretreatment	Pre-culture grown in exponential phase. Inoculum level adjusted to give an initial cell density of 1 x 10^4 cells/ml
Initial cell concentration	Initial cell density – 1x10^4 cells/ml

Table A7_4_1_3-3: Test system

Criteria	Details
Volume of culture flasks	200 ml in 250 ml conical flask
Culturing apparatus	Haemocytometerand microscope
Light quality	White light at 6000 – 10000 lux.
Procedure for suspending algae	Shaking at 200 rpm
Number of vessels/ concentration	6 (3 per concentration)
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_1_3-4: Test conditions

Criteria	Details
Test temperature	20.0°C (incubation temperature)
pH	Start of test 7.4 - 7.5; End of test 7.4 - 7.9
Aeration of dilution water	No
Light intensity	White light – 6000-10000 lux
Photoperiod	continuous

Table A7_4_1_3-5: Cell concentration data

Test-Substance Concentration (nominal/effective)[1] [mg/l]	Cell concentrations (mean values) [cells/ml x 10^4]							
	measured				Percent of control			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
0	1	4.5	26.33	114.89	100	100	100	100
0.10	1	4.33	18.22	71.11	100	96	69	62
0.18	1	4.33	17.89	61.11	100	96	68	53
0.32	1	3.11	9.89	29.22	100	69	38	25
0.56	1	3.56	7.44	20.33	100	79	28	18
1.0	1	1.89	3.89	4.89	100	42	15	4
Temperature [°C]	20.0	20.0	20.0	20.0				
pH	7.4 - 7.5			7.4 - 7.9				

[1] specify, if TS concentrations were nominal or measured

Table A7_4_1_3-6: Validity criteria for algal growth inhibition test according to OECD Guideline 201

	fulfilled	Not fulfilled
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	**YES**	

Criteria for poorly soluble test substances	**YES**	

Substance under test: **DIFENACOUM Technical**

CHEMEX REFERENCE: **SAMPLE: ECO120139**
Study: ENV5792

Test species: ***Selenastrum capricornutum*, strain CCAP 278/4**

Nominal Concentration mg/l.	**0.1**	**0.18**	**0.32**	**0.56**	**1**
	5.3.3 Measured concentration mg/l				
0 hrs fresh	0.09	0.14	0.27	0.49	0.82
72 hrs aged	0.07	0.12	0.19	0.37	0.63
Mean measured Concentration mg/l	**0.08**	**0.13**	**0.23**	**0.43**	**0.73**
% recovery of nominal concentration	80	72	72	77	73

Percent inhibition by biomass integral and growth rate

Mean measured Concentration (mg/l)	**Percent inhibition by biomass integral**		**Percent inhibition by growth rate**	
	48 hours	**72 hours**	**48 hours**	**72 hours**
DMF control	22	36	4	12
0.08	26	35	10	10
0.13	27	41	11	13
0.23	60	71	29	29
0.43	64	78	38	37
0.73	86	93	58	66

EC_{50} values by biomass integral (E_bC_{50} value) and growth rate (E_rC_{50} value) Mean Measured concentration

Period of exposure (hours)	**E_bC_{50} value mg/l**	**E_rC_{50} value mg/l**
0 to 48	0.23	0.58
0 to 72	0.14	0.51

Section A7.4.1.4 Inhibition to microbial activity (aquatic)

Annex Point IIA7.4 Inhibition of activated sludge respiration

			Official use only
		1 REFERENCE	
1.1	**Reference**	Staniland, J (2005) An evaluation of the effect of Difenacoum on the Inhibition of Activated sludge respiration according to OECD 209. Chemex Environmental International Ltd. Ref: ENV7006/120139	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with access to data	PelGar International Ltd. Activa srl	
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**	OECD Guideline 209	
2.2	**GLP**	Yes	
2.3	**Deviations**	Yes, pH not monitored during study.	
		3 MATERIALS AND METHODS	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	ECO120139	
3.1.2	Specification	As given in section 2	
3.1.3	Purity	99.7% (w/w)	
3.1.4	Composition of Product	N/A	
3.1.5	Further relevant properties	GLP samples stored in the dark.	
3.1.6	Method of analysis	N/A	
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	N/A	
3.3	**Reference substance**	3,5-dichlorophenol (3,5-DCP) 97% purity A stock solution of 0.5g/l was prepared in distilled water.	
3.3.1	Method of analysis for reference substance	N/A	
3.4	**Testing procedure**		
3.4.1	Culture medium	Batches of synthetic medium were freshly prepared for each test as described in OECD guideline 209	
3.4.2	Inoculum / test organism	(see table A7_4_1_4-2)	
3.4.3	Test system	(see table A7_4_1_4-3)	

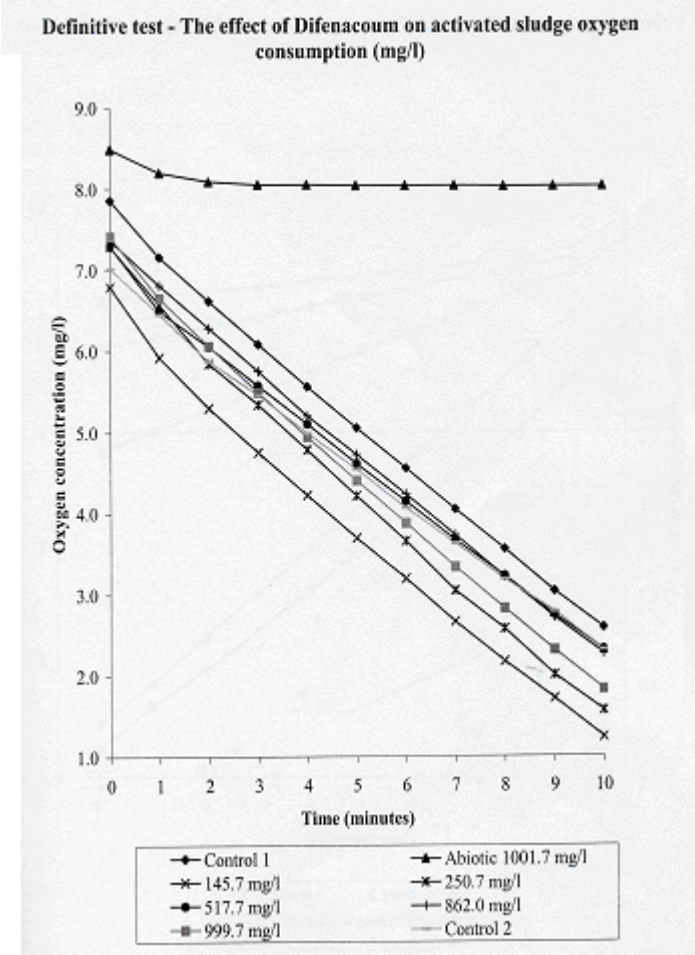
Section A7.4.1.4 Inhibition to microbial activity (aquatic)

Annex Point IIA7.4 Inhibition of activated sludge respiration

3.4.4	Test conditions	(see table A7_4_1_4-4)	
3.4.5	Duration of the test	3 hours	
3.4.6	Test parameter	Respiration inhibition	
3.4.7	Analytical parameter	Oxygen measurement	
3.4.8	Sampling	After a 3 hour incubation period, the concentration of dissolved oxygen (mg/l) was measured every minute in each vessel for 10 minutes.	
3.4.9	Monitoring of TS concentration	No	
3.4.10	Controls	2 controls without test substance were set-up.	
		An abiotic control with the test substance.	
		A reference substance with 3 different test substance concentrations.	
3.4.11	Statistics	N/A	
		4 RESULTS	
4.1 Preliminary test		Not performed	
4.1.1	Concentration	N/A	
4.1.2	Effect data	N/A	
4.2 Results test substance			
4.2.1	Initial concentrations of test substance	N/A	x
4.2.2	Actual concentrations of test substance	N/A	
4.2.3	Growth curves	N/A	
4.2.4	Cell concentration data	N/A	

Section A7.4.1.4 Inhibition to microbial activity (aquatic)

Annex Point IIA7.4 Inhibition of activated sludge respiration

4.2.5	Concentration/ response curve		
4.2.6	Effect data	The EC_{50} value of Difenacoum could not be calculated but is greater than 999.7mg/l	
4.2.7	Other observed effects	None	
4.3	**Results of controls**	Abiotic control indicated there would be no reduction in oxygen concentration in any of test vessels, up to and including, 10001.7 mg/l Difenacoum, attributable to processes in the test system other than those due to the activity of the activated sludge.	x
4.4	**Test with reference substance**	Performed / Not performed	
4.4.1	Concentrations	3,5-DCP at 5, 15 and 30 mg/l	
4.4.2	Results	Estimated graphically to be 5.0 mg/l	

5 APPLICANT'S SUMMARY AND CONCLUSION

Section A7.4.1.4 Inhibition to microbial activity (aquatic)

Annex Point IIA7.4 Inhibition of activated sludge respiration

5.1 Materials and methods		OECD Guideline 209 was followed. A range of test substance concentrations were added to a synthetic sewage medium containing an activated sludge inoculum. Each treatment was placed into a conical flask and vigorously aerated for 3 hour. After 3 hours, the test solution was transferred to a 250ml BOD bottle and the oxygen electrode inserted in such a way as to exclude. The rate of oxygen consumption was measured in each bottle over a 10 minute period . Percentage inhibition of respiration rate was then estimated by comparison with unexposed blanks and a dose-response curve was obtained by plotting percentage inhibition of respiration against exposure concentration of Difenacoum. The sensitivity of the activated sludge micro-organism was assessed by determining the EC_{50} of the reference compound 3,5 DCP during the definitive test.	x
5.2 Results and discussion		Difenacoum did not cause any significant effects on activated sludge respiration inhibition at the concentrations tested, up to and including 999.7mg/l.	
5.2.1	EC_{20}	N/A	
5.2.2	EC_{50}	The EC_{50} value of Difenacoum could not be calculated but is greater than 999.7mg/l	
5.2.3	EC_{80}	N/A	
5.3 Conclusion		The reference compound 3,5-DCP indicated the sensitivity of the activated sludge was within the correct range of 5 to 30mg/l with an EC_{50} OF 5.0 mg/l (estimated graphically). The respiration rates of two blank treatments were within 15% of the mean value.	
5.3.1	Reliability	1	
5.3.2	Deficiencies	No	

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	9.5.2006
Materials and Methods	2.3: pH of the test medium was not measured before start of the experiment. (Monitoring of pH during the test is not required in the OECD 209.). Table A7_4_1_4-2: It is not specified if the sewage treatment plant was treating predominantly domestic sewage. However, in the introduction it is stated that the test was based on the OECD 209 where a sewage treatment plant receiving largely domestic sewage, is used as the microbial source.
Results and discussion	4.2.1: Nominal test concentrations were 61, 145.7, 250.7, 517.7, 862.0 and 999.7 mg/l. The EC50 > 999.7 mg/l. Test concentrations exceeded water solubility of difenacoum at pH 5-9. No estimation were given about water solubility in the test conditions. 4.3: Abiotic control was 1001.7 mg/l. 5.1: 5^{t} row: What should follow exclude?
Conclusion	Difenacoum is not toxic to the activated sludge bacteria at or below water solubility.

Section A7.4.1.4 Inhibition to microbial activity (aquatic)

Annex Point IIA7.4 Inhibition of activated sludge respiration

Reliability	2
Acceptability	Acceptable.
Remarks	-

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A7_4_1_4-1: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Dispersion	N/A
Vehicle	N/A
Concentration of vehicle	N/A
Vehicle control performed	N/A
Other procedures	N/A

Table A7_4_1_4-2: Inoculum / Test organism

Criteria	Details
Nature	Activated sludge
Species	N/A
Strain	N/A
Source	Sewage treatment plant
Sampling site	Cambridge Sewage Treatment Works, Milton Road Cambridge.
Laboratory culture	No
Method of cultivation	N/A
Preparation of inoculum for exposure	The sludge was centrifuged and the pellet re-suspended in dechlorinated tap water.
Pretreatment	N/A
Initial cell concentration	0.60g/l

Table A7_4_1_4-3: Test system

Criteria	Details
Culturing apparatus	BOD flasks
Number of culture flasks/concentration	12 separate 500ml glass conical flasks
Aeration device	Aquarium type air pump
Measuring equipment	Dissolved oxygen meter
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_1_4-4: Test conditions

Criteria	Details
Test temperature	Dilution water was help at approximately 21 +/- 2°C.
pH	N/A
Aeration of dilution water	Yes, vigorously
Suspended solids concentration	N/A

Section A7.4.2 Bioconcentration in aquatic/terrestrial organisms

Annex Point IIA7.5

			Official use only
		1 REFERENCE	
1.1 Reference		Safepharm Laboratories Limited (2004) QSAR method for estimation of bioconcentration factor, EPIWIN v 3.12	
1.2 Data protection		Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with access to data	PelGar International Ltd. Activa srl	
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		Not applicable	
2.2 GLP		Not applicable	
2.3 Deviations		Not applicable	
		3 MATERIALS AND METHODS	
3.1 Test material		Not applicable	
3.1.1	Lot/Batch number	Not applicable	
3.1.2	Specification	Not applicable	
3.1.3	Purity	Not applicable	
3.1.4	Further relevant properties	Not applicable	
3.1.5	Radiolabelling	Not applicable	
3.1.6	Method of analysis	Not applicable	
3.2 Reference substance		Not applicable	
3.2.1	Method of analysis for reference substance	Not applicable	
3.3 Testing/estimation procedure			
3.3.1	Test system/ performance	Not applicable	
3.3.2	Estimation of bioconcentration	Not applicable	
		4 RESULTS	
4.1.1	Concentrations of test material during test	Not applicable	
4.1.2	Bioconcentration factor (BCF)	Not applicable	
4.1.3	Uptake and	Not applicable	

Section A7.4.2 Bioconcentration in aquatic/terrestrial organisms

Annex Point IIA7.5

	depuration rate constants	
4.1.4	Depuration time	Not applicable
4.1.5	Metabolites	Not applicable
4.1.6	Other Observations	Not applicable
4.2 Estimation of bioconcentration		Equation used to make BCF estimate: Log BCF = -1.37 log Kow + 14.4 + Correction value Estimated Log BCF = 3.955 (BCF = 9010)
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.1 Materials and methods		Difenacoum structure was analysed using the QSAR programme, EPIWIN v 3.12 and the results interpreted.
5.2 Results and discussion		Equation used to make BCF estimate: Log BCF = -1.37 log Kow + 14.4 + Correction value Estimated Log BCF = 3.955 (BCF = 9010)
5.3 Conclusion		Estimated Log BCF = 3.955
5.3.1	Reliability	2
5.3.2	Deficiencies	No

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	26.6.2006
Materials and Methods	Agree with the participant.
Results and discussion	BCF = 9010 based on the estimated log K_{ow} of 7.6.
Conclusion	Difenacoum is very likely bioaccumulating in aquatic organisms.
Reliability	Not relevant, not an experimental study.
Acceptability	Acceptable.
Remarks	-

COMMENTS FROM ...

Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Findings	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A7.4.3.3.1 Bioaccumulation in Rainbow trout

Annex Point IIIA XIII.2.3 *(Oncorhynchus mykiss)*

			Official use only
		1 REFERENCE	
1.1 Reference		XXXXX (2004) The Bioconcentration potential of Difenacoum in Rainbow Trout (*Oncorhynchus mykiss*) under flow-through conditions, XXXXX, ENV6596/120139.	
1.2 Data protection		Yes	
1.2.1	Data owner	Activa/PelGar Brodificoum and Difenacoum Task Force	
1.2.2	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, OECD Guidelines for Testing of Chemicals Bioconcentration: Flow-through Fish Test 305.	
2.2 GLP		Yes	
2.3 Deviations		No	x
		3 MATERIALS AND METHODS	
3.1 Test material		As given in section 2	
3.1.1	Lot/Batch number	5907101 / Chemex: ECO120139	
3.1.2	Specification	As given in section 2	
3.1.3	Purity	99.7%	
3.1.4	Further relevant properties	Solubility in water is low. This was not a factor in this study.	
3.1.5	Radiolabelling	Not radiolabelled.	
3.1.6	Method of analysis	Each sample of 4 trout was weighed and blended to a paste using a hand held food blender. 10.0 g of the trout paste was transferred to a flat bottomed dish. 20.0 g of anhydrous sodium sulphate was added and mixed. This mixture was air dired for 24 hours in te dark. The mixture was then reweighed and transferred to an accelerated solvent extraction (ASE) vial. The samples were extracted using an ASE machine with dichloromethane as the solvent (80°C, 1500psi, heat 5 min, flush 75%, cycles x 2). The collected extracts were dried over sodium sulphate and concentrated to 5.0 ml by Kuderna-Danish. 1.0 ml of the extract was removed for lipid determination, the remaining 4.0 ml was passed through a GPC column for cleanup. The GPC column was previously calibrated ising a difenacoum standard. The collected GPC fraction was concentrated to 4.0 ml by Kuderna-Danish. 1.0 ml of the final extract was transferred to an HPLC vial, the remaining 3.0 ml was stored refrigerated in an amber vial. Analysis using HPLC with fluorescence detection was use to measure difenacoum in the extracts. HPLC conditions: Chromatography system: Perkin Elmer Quaternary System HPLC Gradient pump: Perkin Elmer Series 200	x

Section A7.4.3.3.1 Bioaccumulation in Rainbow trout

Annex Point IIIA XIII.2.3 *(Oncorhynchus mykiss)*

		UV detector: Perkin Elmer 785 A UV/VIS @ 254 nm (1.0V/AU) Flourescence detector: Exλ 284 EMλ 390 Interface Box: 900 series and 600 link series Analytical column: Phenomenex Luna 5 µm C18 (2) 250 x 4.6 mm Mobile phase: Methanol: distilled water: acetic acid (850:142:8) Flow rate: 1.5 ml/min Injection volumn: 250 µl The limit of determination was determined as 0.004 µg/l.	
3.2	**Reference substance**	No	
3.2.1	Method of analysis for reference substance	Not applicable.	
3.3	**Testing/estimation procedure**		
3.3.1	Test system/ performance	Eighty rainbow trout were placed in each of three 300 litre polyethylene tanks. The tanks were maintained under through-flow conditions at a volume of 250 litres, one as control and the others at 0.2 µg/l and 0.02 µg/l concentrations of the test substance. The test vessels were maintained at 10.0 ± 1°C. The light source was ceiling mounted fluorescent tubes with a photo period of 16 hours light and 8 hours dark. Observations and records of mortality were made every 24 hours. The feeding rate was 1% body weight per day of Trouw (UK) Ltd Nutra Trout Fry 02 Crumb fish feed. (Quantities were recalculated daily after sampling to adjust for falling fish numbers.) Water samples were taken 24 hours before addition of trout and then at day 0, 1, 3, 10, 15 and 21. The control and exposed trout were also sampled as fish blanks on days: (0), 1, 3, 7, 10, 15, 21, 22, 24, 27, 31 and 35. 500 ml water samples were siphoned from the middle of each tank into an amber glass bottle and stored at 4°C until extraction was completed. Additionally, 4 trout were taken from each vessel and blotted dry. The fish samples were stored frozen (–20 to –35°C) until extraction. Temperature was measured daily to 0.5°C. pH and dissolved oxygen were measured before the addition of the trout, one day after and then weekly during the uptake phase. Measurements of pH (to 0.1) and DO (to 1% ASV) were also recorded at the beginning of the depuration phase, before the addition of the trout and then weekly. Total Organic Carbon (TOC) samples were taken 24 hours before, immediately before trout addition, on day one and then weekly. The uptake phase duration was 21 days and the depuration phase duration was 14 days.	x
3.3.2	Estimation of bioconcentration	Not performed.	
		4 RESULTS	

Section A7.4.3.3.1 Bioaccumulation in Rainbow trout

Annex Point IIIA XIII.2.3 *(Oncorhynchus mykiss)*

4.1 Experimental data

4.1.1 Mortality/behaviour

Cumulative mortalities:

Days from start of test	Exposure Concentration		
	Control	0.2 µg/l	0.02 µg/l
0	0	0	0
1	0	0	0
2	0	0	1
3	0	0	1
4	0	0	1
5	0	0	1
6	0	0	1
7	0	0	1
8	0	0	1
9	0	0	1
10	0	0	1
11	0	0	1
12	0	0	1
13	0	2	1
14	0	2	1
15	0	3	1
16	0	3	1
17	0	4	1
18	0	4	1
19	0	11	1
20	1	15	1
21	1	19	1
22	2	21	1
23	2	22	1
24	2	25	1
25	2	25	1
26	2	25	1
27	2	26	1
28	2	26	1
29	2	26	1
30	2	26	1
31	2	26	1

Section A7.4.3.3.1 Bioaccumulation in Rainbow trout

Annex Point IIIA XIII.2.3 *(Oncorhynchus mykiss)*

	32	2	26	1
	33	2	26	1
	34	2	26	1
	35	2	26	1

4.1.2 Lipid content

Percentage lipid content in fish tissue

Days from start of test	Exposure Concentration		
	Control	0.2 µg/l	0.02 µg/l
0	2.28	-	-
1	2.58	2.22	2.44
3	3.00	4.46	2.62
7	2.24	3.23	2.75
10	2.31	3.16	3.10
15	4.08	3.44	3.92
21	4.07	5.10	4.46
22	3.32	3.62	4.71
24	3.10	4.16	4.44
27	3.62	4.83	4.31
31	3.76	4.61	4.10
35	5.61	5.5	5.64
Average	3.33%	3.88%	3.73%

4.1.3 Concentrations of test material during test

Analysis of difenacoum in water samples was not possible due to problems encountered with the extraction procedure. After a review of the available published literature on difenacoum, the water samples were not analysed as it has been shown that Difenacoum cannot be successfully extracted from water at low concentrations. All results were derived from the nominal concentrations based on flow rates of the dilution water and test material stock solutions.

Difenacoum recovery from fish samples:

Days since test start	Control		0.2 µg/l		0.02 µg/l	
	µg/g fish	µg/g lipid	µg/g fish	µg/g lipid	µg/g fish	µg/g lipid
0	-	-	-	-	-	-
1	-	-	0.0012	54.9	0.0015	61.5
3	-	-	0.0032	72.4	0.0000	0.3
7	-	-	0.0015	45.6	-0.0005	-17.3
10	-	-	0.0296	936.0	0.0021	68.2
15	-	-	0.0227	660.2	0.0067	170.8
21	-	-	0.0194	380.8	0.0099	221.1

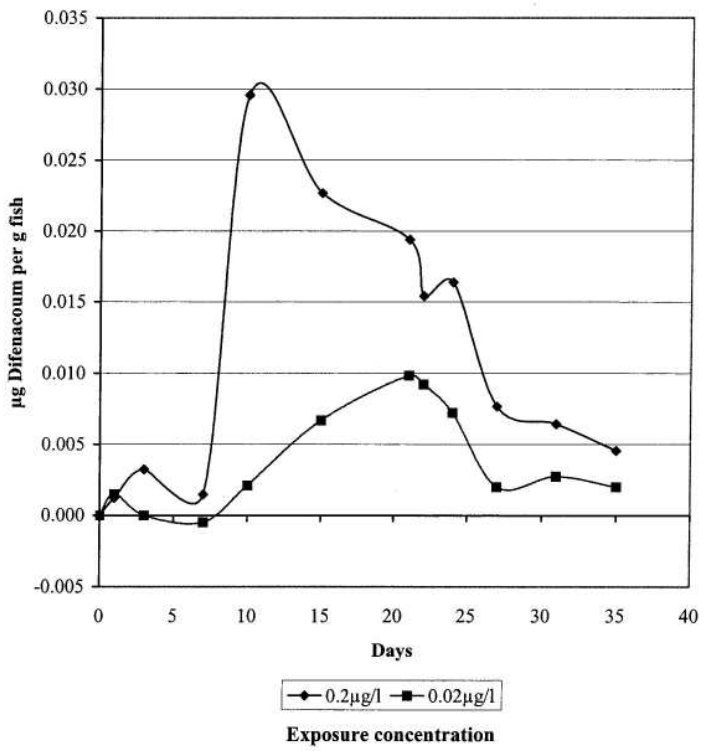
Section A7.4.3.3.1 Bioaccumulation in Rainbow trout

Annex Point IIIA XIII.2.3 *(Oncorhynchus mykiss)*

	22	-	-	0.0154	426.7	0.0092	195.8
	24	-	-	0.0164	369.3	0.0072	233.8
	27	-	-	0.0077	158.9	0.0020	46.5
	31	-	-	0.0064	139.7	0.0027	66.6
	35	-	-	0.0046	82.9	0.0020	35.4

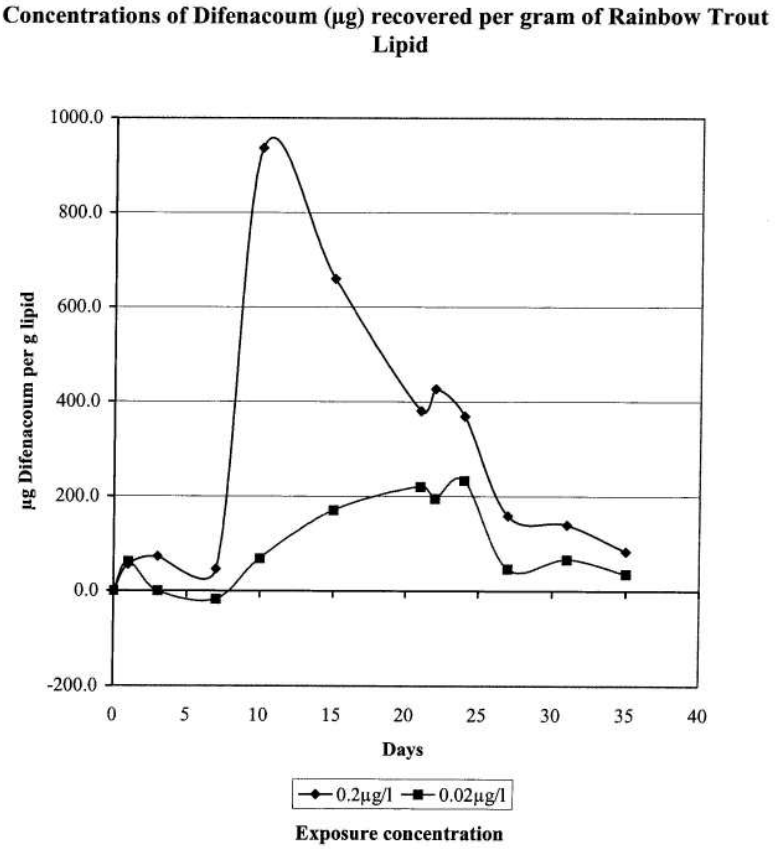
4.1.4

Concentrations of Difenacoum (µg) recovered per gram of Rainbow Trout tissue



Section A7.4.3.3.1 Bioaccumulation in Rainbow trout

Annex Point IIIA XIII.2.3 *(Oncorhynchus mykiss)*



4.1.5	Bioconcentration factor (BCF)	Under the circumstances encountered in this study, (mortalities in the 0.2 µg/l group, leading to early termination of the uptake phase and hence failure to reach steady state,) it was not possible to determine the bioconcentration factor. Calculation (based on prediction formula in OECD 305 guideline) of time to 80% of steady state for difenacoum, was 76.2 days, which exceeds the OECD guideline on maximum uptake phase duration of 60 days.	x
4.1.6	Uptake and depuration rate constants	Distinct uptake and depuration phases were identified from the analysis of the extracts of the 0.02 µg/l group.	x
4.1.7	Depuration time	A reduction of 83% body burden of difenacoum was recorded by the end of the depuration phase (14 days) in Rainbow trout from both test concentrations.	
4.1.8	Metabolites	No metabolites identified.	
4.1.9	Other Observations	Analysis of difenacoum in water samples was not possible due to problems encountered with the extraction procedure. After a review of the available published literature on difenacoum, the water samples were not analysed as it has been shown that Difenacoum cannot be successfully extracted from water at low concentrations. All results were derived from the nominal concentrations based on flow rates of the dilution water and test material stock solutions.	x
4.2	**Estimation of bioconcentration**	Under the circumstances encountered in this study, (mortalities in the 0.2 µg/l group, leading to early termination of the uptake phase and hence failure to reach steady state,) it was not possible to determine the	

Section A7.4.3.3.1 Bioaccumulation in Rainbow trout

Annex Point IIIA XIII.2.3 *(Oncorhynchus mykiss)*

	bioconcentration factor. Calculation (based on prediction formula in OECD 305 guideline) of time to 80% of steady state for difenacoum, was 76.2 days, which exceeds the OECD guideline on maximum uptake phase duration of 60 days.
	5 APPLICANT'S SUMMARY AND CONCLUSION
5.1 Materials and methods	OECD 305 (1996) Analysis of difenacoum in water samples was not possible due to problems encountered with the extraction procedure. The uptake phase was terminated early due to the number of mortalities, hence the steady state of difenacoum in water and Rainbow Trout was not achieved.
5.2 Results and discussion	This study has demonstrated that the test material will accumulate in and depurate from the body tissues of Rainbow trout. A maximum recovery of 0.0296 µg/g difenacoum was recorded in macerated whole fish from the 0.2 µg/l test concentration. Of this accumulated body burden, a reduction of 83% of the difenacoum was recorded by the end of the depuration phase of 14 days. Failure to reach steady state means that the BCF could not be determined from this study. Analysis of difenacoum in water samples was not possible due to problems encountered with the extraction procedure. Difenacoum is an anti-coagulant and will cause bleeding to occur, hence it is possible that difenacoum will be lost from fish tissues.
5.3 Conclusion	This study has demonstrated that the test material will accumulate in and depurate from the body tissues of Rainbow trout. . Of this accumulated body burden, a reduction of 83% of the difenacoum was recorded by the end of the depuration phase of 14 days.
5.3.1 Reliability	1
5.3.2 Deficiencies	No

Evaluation by Competent Authorities

Section A7.4.3.3.1 Bioaccumulation in Rainbow trout

Annex Point IIIA XIII.2.3 *(Oncorhynchus mykiss)*

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	15.5.2006
Materials and Methods	General comments: Serious problem was identified in the test report. The test was started although there was no analytical method available for difenacoum in water in concentration range used in the test. The limit of determination (4 µg/l) was higher than the test concentrations (0.2 and 0.02 µg/l), although the higher test concentration should be ten-fold higher than its detection limit in water. The adequate analytical method is of outmost importance for this test, and if no adequate analytical method is available radiolabelled test substance should have been used. It's further explained in the test report that it was not possible to extract difenacoum from water at low concentrations and references are made to literature. The references are, however, not given in the test report. The extraction method is neither described in the test report. As a consequence of the extraction problem, difenacoum concentrations in water were not measured. The second problem was too high test concentration (0.2 µg/l as more than 70% of fish has died during 35 day uptake phase period. Due to high mortality, the uptake phase was interrupted, against the instructions given in the test guideline. According to the OECD 305, the uptake phase should be continued until steady state is reached or 60 days, whichever is shorter. The test could have be continued as only low mortality was observed in the control and lower difenacoum treatment. Continuation of the uptake phase may have enabled estimation of BCF closer to the steady state phase for the lower concentration. Detailed comments: 2.3: Concentrations in water were not measured, uptake phase was finished before steady-state was reached. 3.1.6: The limit of determination was 0.004 µg/ml, not µg/l. - Length of test species were not given. - Concentration of solvent (1 ml/l) exceeded the concentration recommended (0.1 ml/l) in the OECD 305. - Temperature was reported to be maintained at 10 ± 0.1 °C. The recommended temperature for the rainbow trout is 13-17 °C. The actual remperature during the test ranged from 12.5 to 14.5 °C.
Results and discussion	BCF was not determined. By assuming log K_{ow} of 7.6 (QSAR estimation), 143 day uptake phase is estimated for 95% of steady-state to be reached. The depuration period may be as long as the uptake phase. In this test the uptake phase lasted 21 days and depuration phase 13 days. High mortality was observed at the concentration of 0.2 µg/l, mortality in control and lower test concentration (0.02 µg/l) was 3-6%. Steady increase of difenacoum was not detected at either exposure concentration during the uptake phase. In particular, at higher exposure concentration difenacoum concentration in fish increased sharply during first 10 days, then decreased towards the end of uptake phase, but increased again in the beginning of depuration phase. After one week exposure negative concentration of difenacoum in fish was measured at the exposure concentration of 0.02 µg/l.
Conclusion	The test showed that difenacoum is accumulating in fish, in particular in the fat tissue. After transferring fish to clean water, difenacoum concentrations in fish diminished.

Section A7.4.3.3.1 Bioaccumulation in Rainbow trout

Annex Point IIIA XIII.2.3 *(Oncorhynchus mykiss)*

Reliability	3
Acceptability	Not acceptable. The test concentrations were not measured in water, the uptake phase was finished before the steady-state was reached, unacceptable high mortality occurred at the higher difenacoum concentration.
Remarks	- The copper concentration of the depuration water was 21.9 µg/l.

	COMMENTS FROM ...
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Findings	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A7.5.1.2 Earthworm, acute toxicity test
Annex Point IIIA XIII 3.2

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2005) The toxicity to *Eisenia foetida foetida* of Difenacoum, XXXXX reference: ENV7007/120139	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with Access to data	PelGar International Ltd. Activa srl	
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	
2.2	**Guideline study**	Yes, OECD 207	
2.3	**GLP**	Yes	
2.4	**Deviations**	No	x
		3 METHOD	
3.2	**Test material**	*As given in section 2*	x
3.2.1	Lot/Batch number	Not stated	
3.2.2	Specification	As given in section 2	
3.2.3	Purity	99.7%	
3.2.4	Composition of Product	Not applicable	
3.2.5	Further relevant properties	Not applicable	
3.2.6	Method of analysis	Not stated	
3.3	**Reference substance**		x
3.3.1	Method of analysis for reference substance	Not stated	x
3.4	**Testing procedure**		
3.4.1	Preparation of the test substance	The test substance is the basic structure, which is defined as the test substance and deionised water. *(see table A7_5_1_2-1)*	
3.4.2	Application of the test substance	The test material was applied to the basic structure as a mixture of fine sand and the appropriate quantity of test material. At low test concentrations, the test solution was prepared in an organic solvent carrier (Acetone) and applied to fine sand. The solvent was allowed to vaporise and the resultant test sample/sand mixture added to the basic substrate. The test substrate was homogenised before used.	
3.4.3	Test organisms	*(see table A7_5_1_2-2)*	
3.4.4	Test system	*(see table A7_5_1_2-3)*	x
3.4.5	Test conditions	*(see table A7_5_1_2-4)*	

Section A7.5.1.2 Earthworm, acute toxicity test
Annex Point IIIA XIII 3.2

3.4.6	Test duration	14 days
3.4.7	Test parameter	mortality
3.4.8	Examination	Day 7 and 14
3.4.9	Monitoring of test substance concentration	No
3.4.10	Statistics	The LC_{50} value was estimated and 95% confidence limits calculated using ToxCalc version 5.0 'Comprehensive Toxicity Data Analysis and Database Software'.

4 RESULTS

4.2 Filter paper test		Not performed
4.2.1	Concentration	Not applicable
4.2.2	Number/ percentage of animals showing adverse effects	Not aplicable
4.2.3	Nature of adverse effects	Not aplicable
4.3 Soil test		
4.3.1	Initial concentrations of test substance	318 mg/kg dry weight
4.3.2	Effect data (Mortality)	*(see table A7_5_1_2-5 & see table A7_5_1_2-6)*
4.3.3	Concentration / effect curve	Not provided
4.3.4	Other effects	
4.4 Results of controls		
4.4.1	Mortality	Mortality at 7 days

Test concentration (mg/kg, dry weight)	Number Alive A	B	C	D	Total number dead	Percent mortality
Control	10	10	10	10	0	0
318	10	10	10	10	0	0
557	10	10	10	10	0	0
994	10	10	10	10	0	0

Mortality at 14 days

Test concentration (mg/kg, dry weight)	Number Alive A	B	C	D	Total number dead	Percent mortality

Section A7.5.1.2 Earthworm, acute toxicity test
Annex Point IIIA XIII 3.2

		Control	10	10	10	10	0	0
		318	10	10	10	10	0	0
		557	10	10	0	10	0	0
		994	10	10	10	10	0	0

4.4.2	Number/ percentage of earthworms showing adverse effects	None	
4.4.3	Nature of adverse effects	Not applicable	
4.5	**Test with reference substance**	Performed	
4.5.1	Concentrations	32, 56, 99, 178, 316	
4.5.2	Results	LC_{50} >994 mg/kg dry weight	x

5 APPLICANT'S SUMMARY AND CONCLUSION

5.2 Materials and methods

The study was conducted according to OECD guideline 207. The test vessel were square plastic containers. Each test vessel contained 750g of test substrate (wet weight). Individual worms were then selected from the stock animals. Batches of 10 animals were transferred to the vessels containing the test substrate.

The study was performed in constant light (400 to 800lux), to ensure the worms stay in the medium throughout the test. The study was performed at a temperature of 20 ± 2°C.

Records were made of the numbers of animals observed alive after a 7 day period and again after 14 days. Death was defined by a complete lack of reaction to gentle stimulus applied to the front end of the worm. As dead animals may have decomposed, any missing animals were counted as dead.

Records were kept of any behavioural or pathological abnormalities. At the end of the 14 day exposure period, the moisture content of the test substrate was determined.

5.3 Results and discussion

The highest no-observed effect concentration was estimated as 994 mg/kg dry weight. The lowest observed effect concentration was >994 mg/kg dry weight. The lowest concentration giving 100% was not determined.

0 of the forty control earthworms died during the study.

5.3.1	LC_0	
5.3.2	LC_{50}	> 994 mg/kg dry weight
5.3.3	LC_{100}	

5.4 Conclusion

The LC_{50} was > 994 mg/kg, and the lowest concentration giving 100% mortality was not determined.

5.4.1	Other Conclusions	
5.4.2	Reliability	1
5.4.3	Deficiencies	Yes, The preliminary study was not described, only the results were shown, however this does not effect the integrity of the study

Section A7.5.1.2 Earthworm, acute toxicity test
Annex Point IIIA XIII 3.2

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	11.5.2006
Materials and Methods	2.3: 3 test concentrations instead of 5 recommended in the OECD 207. 3.1.1: Test substance stored 3 years and it is not stated whether the substance was kept away from light. That was, however, recommended in the certificate of analysis (Appendix 1). Difenacoum is rapidly degraded in light, so storage in dark is important. 3.2: Reference substance was 2-chloracetamide. Table A7_5_1_2-3: Amount of artificial soil per container was 750 g wet weight, test concentrations are given in dry weight, number of earthworms per test concentration was 40. 4.2.1: Nominal test concentrations were control, 318, 557 and 994 mg/kg dry weight.
Results and discussion	4.4.2: 13-day LC50 of reference substance (2-chloracetamide) was 194 mg/kg dry weight (95% confidence limits 176-215 mg/kg dry weight). Difenacoum was not toxic to earthworms up to 994 mg/kg dry weight. LC50 could not be determined
Conclusion	Difenacoum is very slightly toxic to earthworms.
Reliability	2
Acceptability	Acceptable
Remarks	-
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A7_5_1_2-1: Preparation of TS solution

Criteria	Details
Type and source of dilution water	Not stated
Alkalinity / Salinity	Not stated
Hardness	Not stated
pH	Start of test = 6.4
Moisture content	Start of test = 45.6 End of test = 43.0
Conductance	Not stated
Holding water different from dilution water	No
In case of the use of an organic solvent	
Dispersion	Yes
Vehicle	Yes, Acetone
Concentration of vehicle	Not stated
Vehicle control performed	Not stated
Other procedures	

Table A7_5_1_1-2: Test organisms

Criteria	Details
Species/strain	*Eisenia foetida foetida*
Source of the initial stock	Blades Biological Ltd, Kent, UK
Culturing techniques	Not stated
Age/weight	Mean Weight: 425 Max: 597 Min: 302 Age: at least 2 months old
Pre-treatment	Temperature = 19.0 to 20.0°C

Table A7_5_1_1-3: Test system

Criteria	Details
Artificial soil test substrate	10% sphagnum peat 20% Kaolin clay 60% industrial fine sand 10% B&Q Organic peat free multipurpose compost About 1% calcium carbonate, pulverised, added to bring the pH between 6.0 and 6.5
Test mixture	750g of test substrate in 2 litres of test vessels containing artificial soil
Size, volume and material of test container	2 litre
Amount of artificial soil (kg)/ container	Not stated
Nominal levels of test concentrations	0, 318, 557, 994 mg/kg
Number of replicates/concentration	4
Number of earthworms/test concentration	10
Number of earthworms/container	10
Light source	artificial
Test performed in closed vessels due to significant volatility of test substrate	No

Table A7_5_1_2-4: Test conditions

Criteria	Details
Test temperature	20 ± 2°C
Moisture content	Start of test = 45.6 End of test = 43.0
pH	Start of test = 6.4
Adjustment of pH	No
Light intensity / photoperiod	400 to 800 lux
Relevant degradation products	Not relevant

Table A7_5_1_2-5: Mortality data

Test Substance Concentration (nominal/measured)[1] [mg/kg artificial soil]	Mortality			
	Number		Percentage	
	7 d	14 d	7 d	14 d
Control	0	0	0	0
318	0	0	0	0
557	0	0	0	0
994	0	0	0	0
Temperature [°C]	20 ± 2°C	20 ± 2°C		
pH	-	-		
Moisture content		43.0		

[1] specify, if TS concentrations were nominal or measured

Table A7_5_1_2-6: Effect data

	14 d [mg/kg soil]	95 % c.l.
LC_0		
LC_{50}	> 994	
LC_{100}		

Table A7_5_1_2-7: Validity criteria for acute earthworm test according to OECD 207

	fulfilled	Not fulfilled
Mortality of control animals < 10%	**x**	

Section 7.5.3.1.1 (3) Acute oral toxicity on birds

Annex Point IIIA XIII 1.1

LD_{50} Difenacoum in Japanese Quails

			Official use only
		1 REFERENCE	
1.1	**Reference**	XXXXX (2005) Acute Oral Toxicity of Difenacoum Technical of the Japanese Quail (Coturnix Coturnix Japonica) XXXXX. Study Code: 04/904-115FU.	**x**
1.2	**Data protection**	Yes	
1.2.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.2.2	Companies with access to data	PelGar International Ltd. Activa srl	
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 authorisation.	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	US EPA Ecological Effects Guidelines, OPPTS 850.2100	
2.2	**GLP**	Yes	
2.3	**Deviations**	None	
		3 METHOD	
3.1	**Test material**	As given in section 2	
3.1.1	Lot/Batch number	03652	
3.1.2	Specification	As given in section 2	
3.1.3	Purity	99.70 % w/w	
3.1.4	Composition of Product	N/A	
3.1.5	Further relevant properties	Must be stored in cool dry place and away from sunlight	
3.1.6	Method of analysis in the diet	N/A	
3.2	**Administration of the test substance**	Corn oil	
3.3	**Reference substance**	No	
3.3.1	Method of analysis for reference substance	N/A	
3.4	**Testing procedure**		
3.4.1	Test organisms	See table A7_5_3_1_1-2	x
3.4.2	Test system	See table A7_5_3_1_2-3	x
3.4.3	Diet		x
3.4.4	Test conditions	Table A7_5_3_1_2-4	
3.4.5	Duration of the test	28 days (14 days pre-observation and 14 days post-observation	
3.4.6	Test parameter	Mortality	

Section 7.5.3.1.1 (3) Acute oral toxicity on birds

Annex Point IIIA XIII 1.1

LD_{50} Difenacoum in Japanese Quails

3.4.7	Examination / Observation	Body weight, food consumption, necropsy and clinical observations were made during the 14 day post treatment period.
3.4.8	Statistics	LD_{50} was calculated by SPSS PC+ statistical software using probit analysis and was determined with 95% confidence limits.

4 RESULTS

4.1	**Limit Test / Range finding test**	Performed
4.1.1	Concentration	0.2, 2, 20, 200, 2000 mg/kg-bw and control
4.1.2	Number/ percentage of animals showing adverse effects	

Dose	Control	0.2	2	20	200	2000
Treated birds	2	2	2	2	2	2
Dead birds	0	0	0	0	1	2

4.1.3	Nature of adverse effects	Mortality
4.2	**Results test substance**	
4.2.1	Applied concentrations	Control (0), 31.1, 62.5, 125.0, 250.0, 500.0 mg/kg-bw
4.2.2	Effect data (Mortality)	Male and female LD_{50} value = 153 mg/kg-bw (confidence limit of 95% 109-217 mg/kg-bw)
4.2.3	Body weight	

Dose Group	Average body weight gain
1	3
2	4
3	5
4	10.4
5	10
6	-

x

4.2.4	Feed consumption	N/A
4.2.5	Concentration / response curve	None
4.2.6	Other effects	In groups 1 and 2 no abnormal behaviour was observed.
4.3	**Results of controls**	
4.3.1	Number/ percentage of animals showing adverse effects	There was no abnormal behaviour observed in the control group.
4.3.2	Nature of adverse effects	N/A
4.4	**Test with reference**	Not performed

Section 7.5.3.1.1 (3) Acute oral toxicity on birds

Annex Point IIIA XIII 1.1

LD_{50} Difenacoum in Japanese Quails

substance		
4.4.1	Concentrations	N/A
4.4.2	Results	N/A

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

OPPTS guidelines 850:2100 were followed. Difenacoum technical was administered orally in a single dose to Japanese quails. This was done oral gavage and using corn oil as a vehicle.

A control and 5 dosing groups each containing 5 animals per sex were dosed and examined in the same way afterwards. All animals were allowed 14 days to acclimatise to the housing conditions (see table 7.5.3.1.2.4) before being treated. All birds were fasted 15 hours before treatment and observed for 14 days after treatment.

31.3, 62.5, 125.0, 250.0, 500 mg/kg-bw were the five dosing levels used and a constant volume of 5 ml/kg-bw was used for all groups.

Clinical observations were made on test animals looking at mortality, signs of toxic behaviour or abnormal behaviour. Observations were made 1 h, 3h, 4,h, and 5h after treatment and daily thereafter.

Body weight measurements were measured on day (-14), (-7), 0, 3, 7 and 14 of the test.

Birds which died during the study were examined. All surviving birds were sacrificed and examined for gross pathological changes at the end of the experiment.

5.2 Results and discussion

Shortly after treatment (within the first 60 mins) acute clinical symptoms were observed: birds sitting fluffed feathers in the following dose levels: 62.5, 125.0 and 250.0 and 500.0 mg/kg-bw. At these dose levels each bird recovered by within 24 hours of the treatment. All birds died at the highest dose levels (500.00 mg/kg-bw) between 4 and 6 days after the treatment. The control animals were symptom-free during the study. The mean body weight gain and the food consumption of the birds did not show any toxicologically important statistically significant changes compared to the control.

During necropsy of birds that died the macroscopic changes observed were those that would be expected with acute circulatory failure (as the cause of death).

The test was considered to have met the validity criteria because the mortality in the control group was below 10 percent at the end of the test.

5.2.1 LD_{50}

Male: 177 mg/kg-bw (confidence limit of 95%: 103-312 mg/kg-bw)

Female: 133 mg/kg-bw (confidence limit of 95%: 75- 238 mg/kg-bw)

Male+Female: 153 mg/kg-bw (confidence limit of 95%: 109-217 mg/kg-bw)

5.3 Conclusion

The test was considered to have met the validity criteria because the mortality in the control group was below 10 percent at the end of the test.

5.3.1 Reliability: 1

5.3.2 Deficiencies: No

Section 7.5.3.1.1 (3) Acute oral toxicity on birds

Annex Point IIIA XIII 1.1

LD_{50} Difenacoum in Japanese Quails

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	26.6.2006
Materials and Methods	3.2: Test substance was administered by gavage. Table A7_5_3_1_2-1 has not been filled. 3.4.1: Japanese Quail has been used as a test species, in the guideline (OPPTS 850.2100) Northern Bobwhite and Mallard are mentioned as test species. 3.4.1, Table A7_5_3_1_2-2: The scientific name of Japanese Quail is *Coturnix coturnix japonica*. 3.4.1, Table A7_5_3_1_2-2: Food consumption during the study was 16.2-20.9 g/animal/day. Food consumption during acclimcation period has not been reported. 3.4.2, Table A7_5_3_1_2-3: Number of birds per pen not recorded in the test report, 1 bird per pen mentioned in this study summary, but source for this information is not mentioned. 3.4.2, Table A7_5_3_1_2-3: In Pre-treatment / acclimation entry should read *14 days acclimatisation with a 15* ***hours*** *fast prior to dosing.* 3.4.3: Diet has been explained in Table A7_5_3_1_2-3. According to Appendix 4 the diet did not include Vitamin K.
Results and discussion	4.1.6: Data reported here does not fully correspond to the data noted in the test report. Average body weight gain (g) from day 0 to day 14 was 3.0-4.0-10.4-8.0-10.0 for males and 7.2-13.8-19.0-13.0-11.0 for females in the treatment groups control-31.3-62.5-125-250-500 mg/kg difenacoum. Table A7_5_3_1_2-5: Mortality data should have reported separately for males and females as it has been done in the test report.
Conclusion	Difenacoum is moderately toxic for Japanese Quail in the acute oral exposure.
Reliability	2
Acceptability	Acceptable
Remarks	1.1: The name of author is Gáty, S.
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A7_5_3_1_2-1: Method of administration of the test substance

Carrier / Vehicle	Details
Water	Yes/No *(If yes, specify: e.g. distilled or deionised water, pH)*
Organic carrier	Yes/No *(If yes, specify: e.g. corn oil, glycol)*
Concentration of the carrier [% v/v]	*Give the concentration*
Other vehicle	*e.g. gelatine capsule*
Function of the carrier / vehicle	*Describe the function (e.g. solvent for test substance, facilitation of uptake and digestion)*

Table A7_5_3_1_2-2: Test animals

Criteria	Details
Species/strain	Japanese quail (Coturnic, coturnix japonica)
Source	Dezso Rokolya, Csavoly, Hungary
Age (in weeks), sex and initial body weight (bw)	At least 16 weeks old at start of treatment
Breeding population	
Amount of food	Daily mean food consumption was between 17.6 and 19.8 g/animal/day for all animals in all study groups
Age at time of first dosing	16 weeks
Health condition / medication	Birds were in apparent good health

Table A7_5_3_1_2-3: Test system

Criteria	Details
Test location	Indoors
Holding pens	Cages, 100 cm x 50 cm. Ceiling height 40 cm. Constructed of galvanised wire
Number of animals	60
Number of animals per pen [cm²/bird]	1
Number of animals per dose	10
Pre-treatment / acclimation	14 days acclimatisation with a 15 fast prior to dosing.
Diet during test	Birds were offered poultry standard diet ad libitum, produced by BABOLNA Ltd, Hungary.
Dosage levels (of test substance)	Control, 31.3 62.5, 125.0, 250, 500 mg/kg-bw. Single dose.
Replicate/dosage level	N/A
Feed dosing method	Gavage
Dosing volume per application	Constant dose volume of 5 ml/kg-bw for all dose groups.
Frequency, duration and method of animal monitoring after dosing	Clinical Observations All test birds were observed during the first 60 minutes after dosing, the 3h, 4h and 5h after the treatment and then once each day for 14 days thereafter. Necropsy The birds that died during the study were examined as soon as possible after death. All surviving birds were sacrificed and examined for gross pathological changes at the end of the study.
Time and intervals of body weight determination	Individual body weights were measured on days (-14), (-7), 0, 3, 7 and 14 of the test.

Table A7_5_3_1_2-4: Test conditions (housing)

Criteria	Details
Test temperature	18.6-24.1°C
Shielding of the animals	Not stated
Ventilation	Not stated
Relative humidity	47-62%
Photoperiod and lighting	8 hours of light per day

Table A7_5_3_1_2-5: Mortality data after test termination

Test substance dosage level [mg/kg bw]	Mortality after test termination (…. days)	
	Total number per dose level	Percentage per dose level
Control (0)	0/10	0
31.3	0/10	0
62.5	1/10	10
125.0	4/10	40
250.0	7/10	70
500.0	10/10	100

Table A7_5_3_1_1-7: Validity criteria for avian acute oral toxicity test according to EPA OPPTS 850.2100

	Fulfilled	Not fulfilled
Mortality of control animals <10%	X	

Section A7.5.5 Bioconcentration, terrestrial

Annex Point IIA VII 7.5

			Official use only
		1 REFERENCE	
1.3	**Reference**	Safepharm Laboratories Limited (2004) QSAR method for estimation of bioconcentration factor, EPIWIN v 3.12	
1.4	**Data protection**	Yes	
1.4.1	Data owner	Activa / PelGar Brodifacoum and Difenacoum Task Force	
1.4.2	Companies with access to data	PelGar International Ltd. Activa srl	
1.4.3	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	
2.4	**Guideline study**	Not applicable	
2.5	**GLP**	Not applicable	
2.6	**Deviations**	Not applicable	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Not applicable	
3.1.1	Lot/Batch number	Not applicable	
3.1.2	Specification	Not applicable	
3.1.3	Purity	Not applicable	
3.1.4	Further relevant properties	Not applicable	
3.2	**Radiolabelling**	Not applicable	
3.2.1	Method of analysis	Not applicable	
3.3	**Reference substance**	Not applicable	
3.3.1	Method of analysis for reference substance	Not applicable	
3.4	**Testing/estimation procedure**		
3.4.1	Test system/ performance	Not applicable	
3.4.2	Estimation of bioconcentration	Not applicable	
		4 RESULTS	

Section A7.5.5 Bioconcentration, terrestrial

Annex Point IIA VII 7.5

4.1	**Concentrations of test material during test**	Not applicable
4.2	**Bioconcentration factor (BCF)**	Not applicable
4.3	**Uptake and depuration rate constants**	Not applicable
4.3.1	Depuration time	Not applicable
4.3.2	Metabolites	Not applicable
4.3.3	Other Observations	Not applicable
4.3.4	Estimation of bioconcentration	Equation used to make BCF estimate: Log BCF = -1.37 log Kow + 14.4 + Correction value Estimated Log BCF = 3.955 (BCF = 9010)

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	Difenacoum structure was analysed using the QSAR programme, EPIWIN v 3.12 and the results interpreted.
5.2	**Results and discussion**	Equation used to make BCF estimate: Log BCF = -1.37 log Kow + 14.4 + Correction value Estimated Log BCF = 3.955 (BCF = 9010)
5.2.1	Conclusion	Estimated Log BCF = 3.955
5.2.2	Reliability	2
5.2.3	Deficiencies	No

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	7.7.2006
Materials and Methods	Eq. 82d from the TGD should be used for the derivation of the terrestrial BCF. Estimated log K_{ow} of 7.6 (US EPA EPIWIN) will be used for the calculation.
Results and discussion	BCF = 477 729
Conclusion	Difenacoum is very likely bioaccumulating in terrestrial organisms.
Reliability	-
Acceptability	-
Remarks	-

COMMENTS FROM ...

Date	*Give date of comments submitted*

Section A7.5.5 Bioconcentration, terrestrial

Annex Point IIA VII 7.5

Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Findings	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	