Section A7.4.1.1 Acute toxicity to fish

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			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted]; Cypermethrin cis:trans/40:60: Acute toxicity to *Oncorhynchus mykiss*; [redacted] Dates of experimental work: 21 April 2005 – 21 July 2005	
1.2	**Data protection**	Yes	
1.2.1	Data owner	Chimac-Agriphar s.a.	
1.2.2			
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, OECD guideline 203 and Annex V to EC Directive 92/69/EEC, Part C1	
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	SL25163S63	
3.1.2	Specification	As given in section 2	
3.1.3	Purity	93.05 % w/w	
3.1.4	Composition of Product	Not applicable, study was carried out on the active substance	
3.1.5	Further relevant properties	Cypermethrin has a very low water solubility and was difficult to maintain at concentrations in the flow through range finding test. For this reason the test design in the main study was changed to semi-static and the test system was preconditioned for 96 hours before the addition of fish.	
3.1.6	Method of analysis	Concentrations of cypermethrin in the test media were determined by Gas Chromatography with Electron Capture detection (GC-ECD) (method ref. CLE(E)1669/022-01V). Each stereoisomer of cypermethrin cis:trans 40:60 was measured individually and the total cypermethrin cis:trans 40:60 concentration was calculated by summing the four individual stereoisomer concentrations. The method was fully validated in a separate study by Covance Laboratories. Cypermethrin was first extracted from the fish dilution water by adding saturated aqueous sodium chloride followed by hexane. The flask was shaken for *ca.* 1 minute and the layers were allowed to separate. The lower aqueous layer was drained into a 1-litre glass bottle and the upper hexane layer was passed through a plug of anhydrous sodium sulphate, into a 150-mL glass bottle. The aqueous layer was returned to the separating funnel. The aqueous sample was extracted	

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		with a further two portions of hexane rinsing the glass bottle which had contained the aqueous layer with hexane each time. The hexane extracts were combined, evaporated to dryness under a stream of nitrogen at 40°C and reconstituted in 1 mL of toluene, with the aid of ultrasound treatment.
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	see table A7_4_1_1-1
3.3	**Reference substance**	No
3.4	**Range Finder**	Two separate range finding tests were performed. The first was performed as a flow through test, at concentrations of 0.1, 1, 10 and 100 µg/L. Due to problems in maintaining the concentrations of test substance under flow-through conditions, the test design was changed to semi-static. The second range-finding test was performed using fewer concentrations to minimise the number of fish used. The concentrations used were 0.01, 1 and 100 µg/L.
3.5	**Testing procedure**	
3.5.1	Dilution water	See table A7_4_1_1-2
3.5.2	Test organisms	See table A7_4_1_1-3
3.5.3	Test system	See table A7_4_1_1-4
3.5.4	Test conditions	See table A7_4_1_1-5
3.5.5	Duration of the test	96 hours
3.5.6	Test parameter	Mortality
3.5.7	Sampling	At 0 and 72 hours, a sample (100 mL) of each freshly prepared test media was taken from each vessel for chemical analysis of cypermethrin *cis:trans* 40:60. Samples (1000 mL) of the freshly prepared control and solvent control were also taken for analysis. At 24 and 96 hours, a sample (100 mL) of old media from each individual vessel was taken for chemical analysis of cypermethrin *cis:trans* 40:60. Samples (1000 mL) of the freshly prepared control and solvent control were also taken for analysis. Samples of the acetone stock solutions used to prepare the test media were also analysed. The 4.01 and 1000 mg/L acetone stock solutions were analysed at the start and end of the test, in order to demonstrate the stability of cypermethrin *cis:trans* 40:60 in acetone. Samples of the remaining acetone stock solutions were analysed at the end of the test only. All extracts were stored refrigerated (1-10ºC) where necessary
3.5.8	Monitoring of TS concentration	Yes, see section 3.4.7 above
3.5.9	Statistics	LC_{50} values were calculated using a Probit method

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4 RESULTS

4.1	**Limit Test**	Not performed
4.2	**Results test substance**	
4.2.1	Initial concentrations of test substance	Nominal cypermethrin concentrations used in the main study were 0.401, 0.882, 1.94, 4.27, 9.39, 20.7, 45.5 and 100 µg/L
4.2.2	Actual concentrations of test substance	See table A7_4_1_1-8
4.2.3	Effect data (Mortality)	See tables A7_4_1_1-9 and A7_4_1_1-10
4.2.4	Concentration / response curve	Not determined
4.2.5	Other effects	After *ca*. 5 hours, it was observed that all of the fish in the 20.7, 45.5 and 100 µg/L test media were displaying signs of pain and distress. Symptoms being displayed included flared opercular, nervous and erratic darting, barrel rolling, immobility, hyperventilation, convulsions/tremors, severe head shaking and loss of normal pigmentation; their bodies were very pale compared to the fish at the lower concentrations and in the control treatments. Therefore, these fish were killed *in extremis*.
4.3	**Results of controls**	
4.3.1	Number/ percentage of animals showing adverse effects	See table A7_4_1_1-9 After 48 hours, in the solvent control treatment, 1 of the fish was found dead outside of the vessel, having escaped through the side of the lid.
4.3.2	Nature of adverse effects	Not applicable
4.4	**Test with reference substance**	Not performed

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	The acute toxicity of cypermethrin cis:trans 40:60 to the Salmonid fish , Onchorhynchus mykiss was determined at approximately 14°C in a 96-hour, semi-static definitive test. The definitive test was conducted at nominal cypermethrin cis:trans 40:60 exposure concentrations of 0.401, 0.882, 1.94, 4.27, 9.39, 20.7,

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45.5 and 100 µg/L, based on the results of two range-finding tests. In the definitive test, initially, a solvent (acetone) stock solution was prepared at a nominal cypermethrin cis:trans 40:60 concentration of 1000 mg/L. A concentration series of solvent (acetone) stock solutions was then prepared at 455, 207, 93.9, 42.7, 19.4, 8.82 and 4.01 mg/L by serial dilution of the 1000 mg/L solvent stock solution.

The test media at nominal cypermethrin cis:trans 40:60 concentrations of 100, 45.5, 20.7, 9.39, 4.27, 1.94, 0.882 and 0.401 µg/L, were prepared by measuring a 1.4 mL aliquot of the appropriate acetone stock solution into a 15-litre constructed glass aquarium (test vessel) part filled with treated mains water. Each test vessel was made up to 14 litres with treated mains water and stirred for homogeneity. Appropriate solvent (1.4 mL of acetone into 15 litres of treated mains water) and dilution water control media (15 litres of treated mains water only) were also prepared.

5.2 Results and discussion

The overall geometric mean measured concentrations of cypermethrin cis:trans 40:60 in the 0.401, 0.882, 1.94, 4.27, 9.39, 20.7, 45.5 and 100 µg/L test media were 0.509, 0.874, 1.944, 4.11, 8.74, 22.2, 44.8 and 79.3 µg/L respectively (corresponding to 127, 99.1, 100, 96.3, 93.1, 107, 98.5 and 79.3% of the nominal concentration). As some values were outside the 80 to 120% of nominal range, the toxicity values were therefore expressed in terms of the geometric mean measured concentrations.

The 24-hour LC_{50} toxicity value was calculated to be 4.26 µg/L (95% CI not determined). The highest concentration at which no mortality occurred was 1.94 µg/L. The lowest concentration at which 100% mortality occurred was 8.74 µg/L.

The 48, 72 and 96-hour LC_{50} toxicity values were all calculated to be 2.83 µg/L (95% CI not determined). The highest concentration at which no mortality occurred was 1.94 µg/L. The lowest concentration at which 100% mortality occurred was 4.11 µg/L.

5.2.1	LC_0	1.94 µg/L
5.2.2	LC_{50}	2.83 µg/L (95% CI not determined)
5.2.3	LC_{100}	4.11 µg/L
5.3	**Conclusion**	The validity criteria of control mortality being less than 10% and maintaining a dissolved oxygen concentration above 60% of the air saturation value were satisfied
5.3.1	Other Conclusions	
5.3.2	Reliability	1
5.3.3	Deficiencies	No

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	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	June 2007
Materials and Methods	Applicant’s version is acceptable.
Results and discussion	Applicant's version is adopted.
Conclusion	Applicant's version is adopted.
Reliability	1
Acceptability	Acceptable
Remarks	Calculated toxicity value, very close to the water solubility value of the active. In the table 7_4_1_1_4, test system: the fish stock density was 1g/l and not 0.8g/l (cf Appendix 1 of the study report).
	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_1-1: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Dispersion	No
Vehicle	Yes, Acetone was used as the vehicle
Concentration of vehicle	A 1000 mg/L stock solution was prepared (0.1009g cypermethrin in 100ml acetone). This was then used to prepare a concentration series of stock solutions of 1000, 455, 207, 93.9, 42.7, 19.4, 8.82 and 4.01 mg/L which were then used to prepare the nominal test media concentrations in the glass aquaria (1.4ml of concentration stock added to 14 L water)
Vehicle control performed	Yes, 1.4ml of acetone alone was added to 15 L water in the solvent control aquarium.
Other procedures	A semi-static test design was used in order to help maintain the appropriate test media concentrations.

Table A7_4_1_1-2: Dilution water – measurements taken in holding tanks

Criteria	Details
Source	De-chlorinated laboratory mains supply.
Alkalainity[1]	27.3 mg/L (HCO_3)
Residual chlorine	0.00-0.06 mg/L
Hardness	79-98 mg/l (as $CaCO_3$)
pH	7.1-7.5
Oxygen content	90-98% (air saturation volume)
Conductance[1]	165 uS/cm
Holding water different from dilution water	No

[1] Not measured in holding tanks, values taken from certificate of analysis for laboratory mains supply water

Table A7_4_1_1-3: Test organisms

Criteria	Details
Species/strain	*Onchorhynchus mykiss*
Source	Recognised external supplier
Wild caught	No
Age/size	Mean fork length of 5.3 cm and mean wet of 1.605g
Kind of food	The fish were fed a proprietary food, which was supplied to the fish in quantities and grades of fineness dependent on the size of the fish. The food was not considered to contain contaminants likely to affect the outcome of the study.
Amount of food	Appropriate to size of fish.
Feeding frequency	Not specified in report
Pre-treatment	Fish were acclimated for at least 12 days
Feeding of animals during test	No

Table A7_4_1_1-4: Test system

Criteria	Details
Test type	Semi static
Renewal of test solution	Two sets of aquaria were used to allow for daily renewal of test solution.
Volume of test vessels	15L-constructed glass aquaria containing 14L test media.
Volume/animal	Fish stock density was 0.80 g/L (weight of fish per litre of test media)
Number of animals/vessel	7
Number of vessels/ concentration	Two sets of vessels were prepared for each concentration but only one set was used at any one time during the test.
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_1_1-5: Test conditions

Criteria	Details
Test temperature	14.1-14.9 ºC, see table A7_4_1_1-6 below
Dissolved oxygen	See table A7_4_1_1-7 below
pH	7.2-7.5, see table A7_4_1_1-6 below
Adjustment of pH	No
Aeration of dilution water	Yes, using an oil free supply of compressed air, bubbled through test media via a glass tube (preconditioned for 96h)
Intensity of irradiation	Not specified in report
Photoperiod	16 hour light, 8 hour dark

Table A7_4_1_1-6: Temperature and pH measurement

Parameter	**Nominal cypermethrin concentration (µg/L)**	**0-hour (new)**	**24-hour (old)**	**24-hour (new)**	**48-hour (old)**	**48-hour (new)**
#Temperature range over the duration of the study (°C)		14.1 - 14.9				
Test media temperature (ºC)	Control	14.3	14.7	14.1	14.7	14.1
	Solvent control	14.2	14.7	14.2	14.7	14.3
	0.401	14.3	14.7	14.2	14.7	14.3
	0.882	14.3	14.7	14.2	14.7	14.3
	1.94	14.3	14.8	14.2	14.7	14.3
	4.27	14.3	14.7	14.2	14.7	-
	9.39	14.3	14.7	-	-	-
	20.7	14.3	14.7	-	-	-
	45.5	14.3	14.7	-	-	-
	100	14.3	14.7	-	-	-
pH	Control	7.4	7.3	7.3	7.5	7.4
	Solvent control	7.4	7.3	7.3	7.5	7.4
	0.401	7.4	7.2	7.3	7.5	7.4
	0.882	7.4	7.3	7.3	7.5	7.4
	1.94	7.4	7.3	7.3	7.4	7.4
	4.27	7.4	7.3	7.3	7.5	-
	9.39	7.4	7.3	-	-	-
	20.7	7.4	7.4	-	-	-
	45.5	7.4	7.3	-	-	-
	100	7.4	7.3	-	-	-
Total hardness (mg/L as $CaCO_3$)		98	-	75	-	75
Residual chlorine (mg/L)		0.00	-	0.04	-	0.06

- Value not required

Temperature range recorded using a digital minimum / maximum thermometer

Table A7_4_1_1-6 (continued): Temperature and pH measurement

Parameter	Nominal cypermethrin concentration (µg/L)	72-hour (old)	72-hour (new)	96-hour (old)
Test media temperature (ºC)	Control	14.7	14.3	14.6
	Solvent control	14.7	14.3	14.6
	0.401	14.7	14.3	14.6
	0.882	14.8	14.3	14.7
	1.94	14.8	14.3	14.8
	4.27	-	-	-
	9.39	-	-	-
	20.7	-	-	-
	45.5	-	-	-
	100	-	-	-
pH	Control	7.3	7.3	7.3
	Solvent control	7.3	7.4	7.3
	0.401	7.3	7.4	7.3
	0.882	7.3	7.4	7.3
	1.94	7.4	7.4	7.3
	4.27	-	-	-
	9.39	-	-	-
	20.7	-	-	-
	45.5	-	-	-
	100	-	-	-
Total hardness (mg/L as $CaCO_3$)		-	73	-
Residual chlorine (mg/L)		-	0.05	-

- Value not required

Table A7_4_1_1-7 Dissolved oxygen measurements in test media

Parameter	**Nominal cypermethrin *cis:trans* 40:60 concentration (µg/L)**	**0-hour (new)**	**24-hour (old)**	**24-hour (new)**	**48-hour (old)**
Dissolved oxygen (% ASV/mg/L)	Control	100/10.37	94/9.71	97/9.74	103/10.43
	Solvent control	99/10.30	94/9.69	91/9.40	94/9.54
	0.401	100/10.36	95/9.75	93/9.51	96/9.82
	0.882	100/10.36	93/9.60	93/9.53	98/10.03
	1.94	98/10.28	97/9.95	92/9.61	92/9.44
	4.27	102/10.42	94/9.71	91/9.67	97/9.95
	9.39	100/10.35	95/9.71	-	-
	20.7	100/10.34	96/9.80	-	-
	45.5	103/10.50	93/9.58	-	-
	100	101/10.41	95/9.76	-	-

- Value not required
% ASV - percentage air saturation value

Parameter	**Nominal cypermethrin *cis:trans* 40:60 concentration (µg/L)**	**48-hour (new)**	**72-hour (old)**	**72-hour (new)**	**96-hour (old)**
Dissolved oxygen (% ASV/mg/L)	Control	102/10.38	92/9.46	99/10.17	93/9.57
	Solvent control	101/10.01	91/9.40	98/10.12	93/9.60
	0.401	101/10.01	92/9.51	96/9.97	93/9.59
	0.882	99/10.00	93/9.59	97/10.04	92/9.48
	1.94	100/9.95	95/9.87	97/10.05	93/9.61
	4.27	-	-	-	-
	9.39	-	-	-	-
	20.7	-	-	-	-
	45.5	-	-	-	-
	100	-	-	-	-

- Value not required
% ASV - percentage air saturation value

Table A7_4_1_1-8 Concentration of test substance during the study

Nominal cypermethrin *cis:trans* 40:60 conc. (µg/L)	Measured cypermethrin *cis:trans* 40:60 concentration (µg/L)				$Mean measured concentration (µg/L)	Mean as % of nominal
	0-hour (new)	24-hour (old)	72-hour (new)	96-hour (old)		
Control	-	-	-	-	N/A	N/A
Solvent Control	-	-	-	-	N/A	N/A
0.401	0.502	0.362	0.702	0.527	0.509	127
0.882	1.22	0.693	0.964	0.717	0.874	99.1
1.94	2.24	1.44	2.23	1.98	1.94	100
4.27	4.74	3.57	#	#	4.11	96.3
9.39	9.25	8.25	#	#	8.74	93.1
20.7	23.5	20.9	*	*	22.2	107
45.5	42.3	47.5	*	*	44.8	98.5
100	80.3	78.3	*	*	79.3	79.3
Fort 0.401	0.887	0.386	0.402	0.430	0.493	123
Fort 0.882	N/A	N/A	0.861	0.935	0.897	102
Fort 1.94	N/A	N/A	2.07	2.08	2.08	107
Fort 9.39	9.61	8.66##	N/A	N/A	9.12	97.1
Fort 100	92.2	84.0	N/A	N/A	88.0	88.0

N/A Not applicable

- Test substance not detected above the limit of determination (0.01 µg/L)

No analysis performed, all fish had died

* No analysis performed, fish had already been killed in extremis

$ Mean value determined as the geometric mean of all measured concentrations. Example: Geometric mean for the 0.401 µg/L media was calculated as: 'Exp ((ln(0.502)+ln(0.362)+ln(0.702)+ln(0.527))/4)'.

Mean of two sample results. The results for the 1.94 and 100 µg/L fortification standards at 24 hours were not acceptable due to non-homogenous preparations. All standards were re-analysed following additional ultrasound treatment (to improve homogeneity), including the 9.39 µg/L fortification standard. The 24-hour value for the 9.39 fortification standard is therefore the geometric mean of the initial result (8.96 µg/L) and the re-analysed sample (8.37 µg/L).

Table A7_4_1_1-9: Mortality data

Nominal cypermethrin *cis:trans* 40:60 conc. (µg/L)	No. of Fish	Observation time and symptom classification *											
		ca. 1.5 hours				4-hour				24-hour			
		a	b	c	d	a	b	c	d	a	b	c	d
Control	7	7	-	-	-	7	-	-	-	7	-	-	-
Solvent control	7	7	-	-	-	7	-	-	-	7	-	-	-
0.401	7	7	-	-	-	6	1	-	-	6	1	-	-
0.882	7	5	2	-	-	3	4	-	-	4	3	-	-
1.94	7	4	3	-	-	4	3	-	-	3	4	-	-
4.27	7	4	2	1	-	2	3	2	-	-	2	2	3
9.39	7	-	3	4	-	-	1	6	-	-	-	-	7
20.7	7	-	1	6	-	-	-	7	-	-	-	-	7
45.5	7	-	-	7	-	-	-	7	-	-	-	-	7
100	7	-	-	7	-	-	-	7	-	-	-	-	7
Nominal cypermethrin *cis:trans* 40:60 conc. (µg/L)	**No. of Fish**	**48 hour**				**72-hour**				**96-hour**			
		a	b	c	d	a	b	c	d	a	b	c	d
Control	7	7	-	-	-	7	-	-	-	7	-	-	-
Solvent control	7	6	-	-	$1	6	-	-	$1	6	-	-	$1
0.401	7	5	2	-	-	5	2	-	-	7	-	-	-
0.882	7	4	3	-	-	3	4	-	-	4	3	-	-
1.94	7	4	3	-	-	1	6	-	-	3	4	-	-
4.27	7	-	-	-	7	-	-	-	7	-	-	-	7
9.39	7	-	-	-	7	-	-	-	7	-	-	-	7
20.7	7	-	-	-	7	-	-	-	7	-	-	-	7
45.5	7	-	-	-	7	-	-	-	7	-	-	-	7
100	7	-	-	-	7	-	-	-	7	-	-	-	7

* Symptom classification

a - No toxic effects

b - Mild toxic effects: swimming normally but exhibiting mild effects e.g. increased cough frequency, swimming position in test vessel different to controls

c - Severe toxic effects: eg swimming abnormally or lying on bottom of tank

d - Fish dead

\- Value not required

$ Fish found dead outside the vessel between 24 and 48-hour timepoints

Table A7_4_1_1-10: Effect data

	24 h [µg/l][1]	95 % c.l.	48, 72, 96 h [µg/l][1]	95 % c.l.
LC_0	1.94	Not determined	1.94	Not determined
LC_{50}	4.26	Not determined	2.83	Not determined
LC_{100}	8.74	Not determined	4.11	Not determined

[1] Effect data are based on the geometric mean measured concentration. 95% C.L. could not be determined.

Table A7_4_1_1-11: Validity criteria for acute fish test according to OECD Guideline 203

	fulfilled	Not fulfilled
Mortality of control animals <10%	**Y**	
Concentration of dissolved oxygen in all test vessels > 60% saturation	**Y**	
Concentration of test substance ≥80% of initial concentration during test		**Results based on geometric mean measured concentration**
Criteria for poorly soluble test substances	**Y**	