Section 7.4.3.2 Effects on reproduction and growth rate of fish

Annex Point IIIA XIII 2.2

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted]; Cypermethrin cis:trans/40:60 Fathead Minnow, Early Life Stage test; [redacted] Dates of experimental work: 29 July 2005 – 18 December 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 210 (July 1992) OPPTS 850.1400	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	X
		3 METHOD	
3.1	**Test material**	As given in section 2 (non-radiolabelled cypermethrin) [^{14}C-cyclopropyl]-Cypermethrin-*cis*, specific activity 127.31 µCi/mg [^{14}C-cyclopropyl]-Cypermethrin-*trans*, specific activity 127.23 µCi/mg	
3.1.1	Lot/Batch number	25163S63 (non-radiolabelled cypermethrin)	
3.1.2	Specification	As given in section 2 (non-radiolabelled cypermethrin)	
3.1.3	Purity	93.05% w/w (non-radiolabelled cypermethrin)	
3.1.4	Composition of Product	Not applicable, test carried out on active substance	
3.1.5	Further relevant properties	Low water solubility, therefore test was a continuous flow design.	
3.1.6	Method of analysis	Determination of radioactivity in water samples determined by LSC.	

Section 7.4.3.2 Effects on reproduction and growth rate of fish
Annex Point IIIA XIII 2.2

3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	A stock solution of [^{14}C]-Cypermethrin was prepared in the ratio of 40:60 cis:trans in hexane. This stock solution was maintained at *ca* -20°C and was used to prepare dose stock solutions throughout the study. To ensure an adequate supply of test item for the duration of the study, test concentrations of 1 and 0.32 µg/L were radio-diluted using the supplied non-radiolabelled technical Cypermethrin. The dose stock solutions of [^{14}C]-Cypermethrin were prepared for each test concentration by adding an aliquot (200 µL for test concentrations of 1, 0.32 and 0.1 µg/L, 64 µL for test concentration of 0.032 µg/L and 20 µL for test concentration of 0.01 µg/L) of the [^{14}C]-Cypermethrin stock solution into glass scintillation vials. The hexane was blown to dryness using nitrogen. For test concentrations of 1 and 0.32 µg/L nominal an aliquot (203 µL and 50 µL respectively) of non-radiolabelled technical Cypermethrin (prepared as a 1 mg/mL solution in hexane) was added. This was blown to dryness using nitrogen. Acetone (22.5 mL) was added to each vial and the vials ultrasonicated for *ca* 5-10 min to ensure dissolution. The stock solutions in acetone were stored at *ca* 4°C. Stock solution was delivered to the appropriate mixing vessel at a rate of 0.19 mL/h where it was mixed with dilution water and delivered at a rate of *ca* 15.6 mL/min to each replicate tank. Stock solutions were replenished at ≤96 h intervals throughout the test period as the test item was shown to be stable over 96 h.
3.3	**Reference substance**	No
3.3.1	Method of analysis for reference substance	Not applicable
3.4	**Testing procedure**	
3.4.1	Dilution water	Test and culture water was reconstituted fresh water prepared by the test laboratory (see table A7_4_3_2-1 for recent analysis results)
3.4.2	Test organisms	See table A7_4_3_2-2
3.4.3	Test system	See table A7_4_3_2-3
3.4.4	Test conditions	See table A7_4_3_2-4
3.4.5	Duration of the test	28 days post hatch
3.4.6	Test parameter(s)	Numbers of live and dead embryos recorded daily, with any dead embryos being removed. Mortality, appearance, behaviour and abnormal effects were recorded if noted. Surviving fry counted at day 28 post-hatch and individually weighed and lengths recorded.
3.4.7	Examination / Sampling	Daily inspection of the embryos The temperature in one replicate control tank was measured continuously and in all tanks twice weekly. Dissolved oxygen and pH were measured in a sample of water taken from each tank once weekly. Conductivity, hardness and alkalinity of the dilution water were recorded once weekly.

Section 7.4.3.2 Effects on reproduction and growth rate of fish
Annex Point IIIA XIII 2.2

3.4.8	Monitoring of TS concentration	Yes On each day of the study, commencing at 48 h prior to the addition of the embryos, single aliquots (10 mL) of water were sampled from each replicate solvent control and test item tank and mixed with scintillation fluid (Zinsser Analytic) prior to determination of radioactive content by Liquid Scintillation Counting (LSC). These were subjected to 1 min analysis as a system check. On Days -2, -1, 0, 1, 3, 7, 10, 21, 28 and 31 duplicate samples (single samples on Day -2 and -1) were subjected to 5 min analysis (1 min. analysis for day -2). The samples taken on Days -1, 0, 1 and 3 for 5 min analysis were prepared using Aquasafe as the scintillant. This was subsequently shown to be unreliable and all further analysis was conducted using Quicksafe as the scintillant. In order to obtain reliable Day 0 analysis and generate measured test concentrations, extracts of the Day 0 samples were used. Radioactivity was quantified using a liquid scintillation analyser (Packard 2100 TR) with automatic quench correction by external standard-channels radio. A background count rate was determined prior to analysis and subtracted from each count rate with a limit of reliable determination of 30 dpm. A 1 L sample was removed from replicate test tank A at 1 µg/L and at 0.032 µg/L on study day -2. Similarly, 1 L samples were removed from each test and solvent control tank on study days 0, 14 and 31. Samples were extracted by partitioning with hexane (50ml) and allowing the layers to separate. The hexane layer was then passed through granular sodium sulphate into a collection vessel and the aqueous layer partitioned twice more with hexane and the resulting extracts combined. The extracted samples were analysed for the presence of the parent material by HPLC with UV detection at 280 nm (Spherisorb 5 µm silica 250 x 4.6 mm, flow rate 1 ml/min).
3.4.9	Statistics	2-tailed Fisher's Exact test was used to determine cumulative mortality and number of fry that hatched compared to the controls. Levine's test and ANOVA techniques were used for weight and length data.
		4 RESULTS
4.1	**Range finding test**	Not performed, test concentrations decided based on published information on the toxicity of cypermethrin to fathead minnows.
4.2	**Results test substance**	
4.2.1	Initial concentrations of test substance	0 (solvent and non-solvent controls), 0.01, 0.032, 0.1, 0.32 and 1 µg/L (nominal concentrations)
4.2.2	Actual concentrations of test substance	See Table A7_4_3_2-5

Section 7.4.3.2 Effects on reproduction and growth rate of fish
Annex Point IIIA XIII 2.2

4.2.3 Effect data

Hatching success - results indicated a significantly lower hatching success at 0.032, 0.1 and 0.32 µg/L nominal, when compared to the solvent control. There was however no statistically significant difference in hatching success between all other groups, including 1 µg/L nominal and the solvent control. As a result it was not possible to statistically determine a NOEC with respect to hatching success. It is considered that this non-monotonic response does not indicate an effect of the test item but was rather a biological effect. On study day 2, a significant number of dead/fungus-covered embryos were removed from test concentrations of 0.1 and 0.32 µg/L which resulted in a lower hatching success at these test concentrations. An empirically derived NOEC would be regarded as 1 µg/L nominal (0.89 µg/L based on mean measured concentrations).

Fry survival - results indicated no significant difference in mortality between the 0.01 to 0.32 µg/L treated groups and the solvent control. The NOEC for fry survival is therefore concluded to be 0.32 µg/L nominal (0.30 µg/L mean measured), with the LOEC being 1 µg/L nominal (0.89 µg/L mean measured).

Fry growth (length) - Body length of surviving fry was found to be significantly greater in all treated groups when compared to the solvent control. This may be explained by the number of fry present in each tank. The solvent control replicates contained significantly more fry than all other tanks thus increasing competition for both food and space, resulting in slightly slower growth. The NOEC for deleterious effects on length was calculated as 0.32 µg/L nominal (0.30 µg/L mean measured) with the LOEC being 1 µg/L.

Fry growth (weight) - Body weight of surviving fry was found to be significantly greater in all treated groups when compared to the solvent control. This may be explained by the number of fry present in each tank. The solvent control replicates contained significantly more fry than all other tanks thus increasing competition for both food and space, resulting in slightly slower growth. The NOEC for deleterious effects on body weight was calculated as 0.32 µg/L nominal (0.30 µg/L mean measured) with the LOEC being 1 µg/L.

See Tables A7_4_3_2-6 to A7_4_3_2-10

4.2.4 Other effects

It was observed on Study Day 4 that all fry at 1 µg/L nominal appeared smaller and weaker than fry in all other tanks. They were observed to be swimming in spiralling movements with periods when they appeared to go into spasms. All fry at 1 µg/L nominal (0.89 µg/L mean measured) were dead by post-hatch Day 11.

It was noted on Study Day 8 that in each replicate tank (with the exception of replicate tank B at 0.32 µg/L nominal) there were numbers of smaller and weaker fry. From Study Day 21 to the end of the test these smaller and weaker fry began to undergo significant mortality. All mortality from Study Day 21 to the end on the test was of these smaller and weaker fry.

Section 7.4.3.2 Effects on reproduction and growth rate of fish
Annex Point IIIA XIII 2.2

4.3	**Results of controls**	
4.3.1	Number/ percentage of animals showing adverse effects	Hatching success in the solvent control group was 77% and fry survival 73%, showing the test was valid.
4.4	**Test with reference substance**	Not performed

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	The effect of prolonged exposure to Cypermethrin in the cis:trans ratio of 40:60, on the early-life stages of the Fathead Minnow (*Pimephales promelas)* was assessed over embryo development, hatching and for 28 days post-hatch, in accordance with OECD (July 1992) Guideline 210 and OPPTS 850.1400 Fish Early-Life Stage Test. The test was conducted under continuous flow conditions, with embryos (less than 25 h old on exposure to the test solutions) and larvae/fry exposed to the following nominal concentrations of [^{14}C]-Cypermethrin; 0.01, 0.032, 0.1, 0.32 and 1 µg/L. Both solvent and non-solvent controls were included in the test. Duplicate tanks were tested at each concentration.
5.2	**Results and discussion**	
5.2.1	NOEC	Hatching success = 1 µg/L nominal (0.89 µg/L measured) Fry survival = 0.32 µg/L nominal (0.30 µg/L measured) Fry growth = 0.32 µg/L nominal (0.30 µg/L measured)
5.2.2	LOEC	Fry survival = 1 µg/L nominal (0.89 µg/L measured) Fry growth (length) = 1 µg/L nominal (0.89 µg/L measured) Fry growth (weight) = 1 µg/L nominal (0.89 µg/L measured)
5.3	**Conclusion**	See table A7_4_3_2-11
5.3.1	Other Conclusions	
5.3.2	Reliability	1
5.3.3	Deficiencies	No

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	November 2007

Section 7.4.3.2 Effects on reproduction and growth rate of fish
Annex Point IIIA XIII 2.2

Materials and Methods	Applicant's version is acceptable. 2.3 Deviations: In error, 20 embryos were added in replicate A of solvent test control instead of 0.01µg/L replicate B.
Results and discussion	Applicant's version is acceptable.
Conclusion	Applicant's version is adpoted
Reliability	1
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_4_3_2-1: Dilution water

Criteria	Details
Source	Reconstituted fresh water prepared by the test laboratory.
Chloride	47 mg Cl/L
Sodium	5 mg Na/L
Potassium	<1 mg K/L
Magnesium	4 mg Mg/L
Hardness	80 mg $CaCO_3$
pH	6.66 as $CaCO_3$
Alkalinity	7 mg/L
Conductance	190 µS/cm at 20 ºC
Holding water different from dilution water	No

Table A7_4_3_2-2: Test organisms

Criteria	Details
Species/strain	Fathead Minnow *(Pimephales promelas)*
Source	[redacted]
Wild caught	No
Age/size	25 hours
Pre-treatment	Embryos were randomly selected and added in small groups to the incubation chambers in order from the untreated control to the highest test concentration. This procedure of addition of embryos to the incubation chambers was to avoid possible contamination which may have occurred if embryos had been added randomly over the test concentrations.
Kind of food	Brine Shrimp *(Artemia salina)*
Amount of food	*Ad libitum*
Feeding frequency	At post hatch (day 0), then twice daily for the remainder of the test. Fry were not fed in the 24 hour period before termination of the test.

Table A7_4_3_2-3: Test system

Criteria	Details
Test type	Flow-through
Renewal of test solution	Approx. 15.6 ml/min
Volume of test vessels	22 x 15 x 12cm (3L capacity) containing an 8cm glass egg incubation chamber
Number of animals/vessel	Between 30 and 76 embryos.
Number of vessels/ concentration	Two replicate test tanks at each concentration
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_3_2-4: Test conditions

Criteria	Details
Test temperature	23-27ºC
Aeration of dilution water	A magnetic stirrer was placed in each tank to ensure adequate movement of surrounding water and to maintain suitable dissolved oxygen concentrations in the egg incubation chamber.
Photoperiod	Subdued lighting for the first 3 days after embryo introduction, then 16h light: 8 hour dark with 30 min dawn/dusk transition

Table A7_4_3_2-5: Measured concentration of cypermethrin during the test

Study Day	Nominal Concentration of Cypermethrin (µg/L)					
	0 (Solvent)	0.01	0.032	0.1	0.32	1
-2	0	0.006*	0.038	0.11	0.47	3.88
0	-	0.011	0.028	0.07	0.24	0.60
7	0	0.010*	0.026	0.11	0.33	0.97
10	0	0.006*	0.031	0.10	0.41	1.11
14	0	0.010*	0.034	0.11	0.30	NA
21	0	0.010*	0.034	0.10	0.22	NA
28	0	0.004*	0.030	0.10	0.35	NA
31	0	0.008*	0.030	0.11	0.28	NA
Overall Mean**	0	0.008*	0.030	0.10	0.30	0.89

NA = Not applicable as all fry died earlier.
* = Level of radioactivity below the limit of reliable determination.
** = Overall mean is mean of concentrations on Days 0-31.
\- = Extracted sample not analysed.

Table A7_4_3_2-6: Hatchability and survival data

Conc. µg/L		Initial no. of embryos	No.of Fry		Hatch (%)		Adjusted no. fry/tank	No. fry surviving at 28 d	Survival of Fry from release to 28 Days Post-Hatch (%)	
			Hatched	Released	Individual	Pooled			Individual	Pooled
0	A	55	35	32	64	68	35	19	54	68
	B	55	39	37	71		34	28	82	
0 Solvent	A	76*	60	54	79	77	50	34	68	73
	B	55	41	41	75		45	35	78	
0.01	A	55	40	39	73	68	29	23	79	74
	B	30	19	19	63		29	20	69	
0.032	A	42	26	26	62	60	26	18	69	74
	B	42	24	24	57		24	19	79	
0.1	A	43	19	19	44	38	19	17	89	80
	B	42	13	10	31		10	7	70	
0.32	A	42	18	15	43	38	15	12	80	80
	B	43	14	10	33		10	8	80	
1	A	53	27	23	51	68	23	0	0	0
	B	55	46	46	84		46	0	0	

* 20 embryos added in error

Table A7_4_3_2-7: Fry mortality (no. of dead fry)

Study Day	Nominal Concentration of Cypermethrin (µg/L)													
	0		0 Solvent		0.01		0.032		0.1		0.32		1	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
2	0	0	6*	0	0	0	0	0	0	0	0	0	0	0
3	3	2	0	0	1	0	0	0	0	3	3	4	4	0
4	1	0	2	0	0	0	0	0	0	1	2	1	1	0
5	1	0	2	0	0	0	0	0	1	1	0	0	8	9
6	1	1	0	2	3	2	3	1	0	0	1	0	9	35
7	0	0	0	0	0	1	0	1	1	0	0	0	3	1
8	0	0	0	0	0	0	0	0	0	0	0	1	1	1
9	0	0	0	0	0	0	0	0	0	0	0	0	0	-
10	0	0	0	0	0	0	0	0	0	0	0	0	0	-
11	0	0	0	0	0	0	0	0	0	0	0	0	1	-
12	0	0	0	0	0	0	0	0	0	0	0	0	-	-
13	0	0	0	0	0	0	0	0	0	0	0	0	-	-
14	1	0	0	0	0	0	0	0	0	0	0	0	-	-
15	0	0	0	0	0	0	0	0	0	0	0	0	-	-
16	0	0	0	0	0	0	0	0	0	0	0	0	-	-
17	0	0	0	0	0	0	0	0	0	0	0	0	-	-
18	0	0	0	0	0	0	0	0	0	0	0	0	-	-
19	1^	0	0	0	0	1^	0	0	0	0	0	0	-	-
20	0	0	0	0	0	0	0	0	0	0	0	0	-	-
21	0	0	0	0	0	0	0	0	0	1	0	0	-	-
22	1	1	0	0	0	0	1	0	0	0	0	0	-	-
23	1	0	1	3	0	0	0	0	0	0	0	0	-	-
24	0	0	5	3	0	1	2	1	0	0	0	0	-	-
25	1	0	4	0	4	0	0	0	0	0	0	0	-	-
26	0	1	0	0	0	0	0	0	0	0	0	0	-	-
27	2	0	0	0	0	0	0	0	0	0	0	0	-	-
28	1	2	0	0	0	0	0	0	0	0	0	0	-	-
29	1	1	0	0	0	0	0	0	0	0	0	0	-	-
30	0	0	0	0	0	1	0	0	0	0	0	0	-	-
31	0	0	0	0	0	0	0	0	0	0	0	0	-	-

* Dead larvae possibly due to movement created by magnetic stirrer beneath the egg chamber
^ Fry removed due to ill health
- All fry dead

Table A7_4_3_2-8: Fry growth (length in mm)

	Cypermethrin (µg/L)											
	0		0 solvent		0.01		0.032		0.1		0.32	
	A	B	A	B	A	B	A	B	A	B	A	B
Minimum	17.7	17.2	16.8	17.8	16.5	18.0	18.6	19.6*	16.3*	21.6*	18.8*	20.3
Maximum	22.3	22.3	21.0	20.7	21.9	22.3	22.1	22.9	22.4	23.3	22.8	23.7
Mean	20.2	19.8	18.6	19.1	19.4	19.8	20.3	20.9*	19.9*	22.1*	20.7*	22.4
Standard Deviation	1.10	0.99	1.04	0.75	1.42	1.13	1.16	0.98*	1.76*	0.92*	1.22*	1.20

* Excluding outliers

Table A7_4_3_2-9: Fry growth (weight in mg)

	Cypermethrin (µg/L)											
	0		0 solvent		0.01		0.032		0.1		0.32	
	A	B	A	B	A	B	A	B	A	B	A	B
Minimum	79.0	67.7	58.9	71.2	63.8	89.1	96.3	6.2	5.7	12.1	8.2	132.2
Maximum	163.4	143.7	123.1	132.7	158.7	175.2	158.1	171.5	165.2	213.6	175.5	212.4
Mean	122.8	110.9	89.9	95.6	102.8	123.9	122.6	119.6	105.7	131.5	111.1	174.3
Standard Deviation	20.68	17.65	15.51	13.97	22.83	23.82	21.11	52.16	56.41	83.18	62.55	28.26

Table A7_4_3_2-10: Summary of Effect data

	Parameter	NOEC	LOEC
Data based on nominal cypermethrin concentrations	Hatch	1 µg/L*	*
	Survival	0.32 µg/L	1 µg/L
	Length	0.32 µg/L^	1 µg/L
	Weight	0.32 µg/L^	1 µg/L
Data based on measured cypermethrin concentrations	Hatch	0.89 µg/L	*
	Survival	0.30 µg/L	0.89 µg/L
	Length	0.30 µg/L^	0.89 µg/L
	Weight	0.30 µg/L^	0.89 µg/L

* = No effect was noted on hatching success at 1 µg/L nominal (0.89 µg/L measured). Effect on hatching success was detected at 0.032, 0.10 and 0.32 µg/L nominal (0.0030, 0.10 and 0.30 µg/L measured)

^ = Length and weight of surviving fry was significantly greater at all test concentrations when compared to solvent control. NOEC values are for deleterious effects.

Table A7_4_3_2-11: Validity criteria for fish tests according to OECD Guidelines 210

	fulfilled	Not fulfilled
Hatching success of solvent control group >66%	Y	
Fry survival in solvent control >70%	Y	
Concentration of dissolved oxygen in all test vessels >60%	Y	
Test substance concentrations maintained within ± 20% of mean measured values	Y	