Section A7.4.1.1 Acute toxicity to fish (02)

Annex Point IIA VII.7.1 *Cyprinodon variegatus* (Sheepshead minnow)

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] (2005): Preventol A 4-S. Fish (Sheepshead Minnow), Acute Fish Toxicity Test, Static, 96 h. [redacted], Report-No. FAS91231, Project-No. [redacted], date: 2004-08-19.	X
1.2	**Data protection**	Yes	
1.2.1	Data owner	Lanxess Deutschland GmbH	
1.2.2	Companies with letter of access	-	
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/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes US EPA Subdivision E, § 72.3 (1986) EPA OPPTS Draft Guideline 850.1075 (1996) OECD-Guideline No. 203 for Testing of Chemicals (1992)	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Preventol A 4-S	
3.1.1	Lot/Batch number	Batch number: [redacted]	
3.1.2	Specification	As given in section 2 of dossier	
3.1.3	Purity	[redacted] (HPLC)	
3.1.4	Composition of Product	Dichlofluanid is the active ingredient of Preventol A 4-S (content: [redacted] a.i.)	
3.1.5	Further relevant properties	The test item is known to be hydrolytically not stable under exposure conditions: $t_{1/2}$ = 1.2 h at pH = 8.2, 20 °C. The water solubility was reported to be 1.3 mg/l at 20 °C. Dichlofluanid is non-volatile. pH–value in water = 10.7	
3.1.6	Method of analysis	Dichlofluanid and DMSA were analysed on a reverse phase HPLC – DAD using external standards, method validated.	
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	With regard to the low solubility of the test item and the low amounts needed for the preparation of test item solutions a stock solution and 5 dilution levels in a geometrical series with a dilution factor 2 were prepared: 1:16 - 1:8 - 1:4 - 1:2 - 1:1.	
3.3	**Reference substance**	No	
3.3.1	Method of analysis	-	

	for reference substance	
3.4	**Testing procedure**	
3.4.1	Dilution water	See Table A7_4_1_1-1 (prepared seawater)
3.4.2	Test organisms	See Table A7_4_1_1-2
3.4.3	Test system	See Table A7_4_1_1-3
3.4.4	Test conditions	See Table A7_4_1_1-4
3.4.5	Duration of the test	96 hours
3.4.6	Test parameter	Mortality and behaviour. Fish were dead if there was no visible movement (e.g. gill covers movement) and if touching of the caudal peduncle produced no reaction. considered Records were kept of visible abnormalities (e.g. loss of equilibrium, swimming behaviour, respiratory function, pigmentation, etc.).
3.4.7	Sampling	Biological observations were made after 6, 24, 48, 72 and 96 h. Water quality parameters of temperature, pH-value, dissolved oxygen concentration and salinity were measured at 0, 24, 48, 72 and 96 h. The parameters were determined to be within the acceptable limits. Temperature in the control vessel was measured and recorded continuously (every 30 minutes). Residual chlorine of the water was determined at the beginning of the test out of the control.
3.4.8	Monitoring of TS concentration	The concentrations of the active ingredient dichlofluanid and its metabolite DMSA were determined after 0, 2, 4, 24 and 96 h for all concentrations and control. Water samples were taken from the centre of the aquaria for determination of the concentration of dichlofluanid and its metabolite.
3.4.9	Statistics	LC_{50}-values and confidence intervals after 6, 24, 48, 72 and 96 h were calculated by probit analysis. Probit values according to WEBER (1986) were used. Calculation of the confidence intervals for LC_{50} was carried out using standard procedures according to BREITIG & TÜMPLING (1982).
		4 RESULTS
4.1	**Limit Test**	Not performed
4.1.1	Concentration	-
4.1.2	Number/ percentage of animals showing adverse effects	-
4.1.3	Nature of adverse effects	-

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4.2	**Results test substance**		
4.2.1	Initial concentrations of test substance	The chosen test solutions were selected on the basis of a static range finding test. Nominal concentrations are not given, instead, the initially measured total concentrations were reported in the results (see next chapter).	
4.2.2	Actual concentrations of test substance	The dichlofluanid concentrations decreased markedly within 24 h, whereas increasing concentrations of the metabolite DMSA were found. The recovery rates based on the sum of the results gained for the active ingredient dichlofluanid and the metabolite DMSA (expressed as dichlofluanid equivalents) exceed 80 % after 2, 4, 24 and 96 h compared to the respective initial concentration, except for the initial concentration 54.7 µg/L after 4 h. Furthermore, the sum of the initially measured concentrations of the active ingredient dichlofluanid and its metabolite DMSA (expressed as dichlofluanid equivalents) is called total concentration. Measured concentrations (initially measured total concentrations) were: 54.7 – 1 11 - 230 - 610 - 790 µg/L. See Table A7_4_1_1-5. All effect levels are given based on the total concentration. Additionally, the effect levels are presented based on initially measured concentrations of the a.i. dichlofluanid.	X
4.2.3	Effect data (Mortality)	See Table A7_4_1_1-6 and Table A7_4_1_1-7	
4.2.4	Concentration / response curve	A concentration/ response curve is given in the report (p. 21)	
4.2.5	Other effects	See Table A7_4_1_1-6 for detailed description of observed responses	
4.3	**Results of controls**		
4.3.1	Number/ percentage of animals showing adverse effects	There were neither mortalities nor symptoms of intoxication in the control group	
4.3.2	Nature of adverse effects	-	
4.4	**Test with reference substance**	Not performed	
4.4.1	Concentrations	-	
4.4.2	Results	-	

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	The acute toxicity of the test item Preventol A 4-S ([redacted] dichlofluanid) to fish (Sheepshead minnow) was determined in a static test system according to several current test guidelines.

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5.2	**Results and discussion**	Dichlofluanid was known to be hydrolytically not stable under exposure conditions. The half life of the test item within the performed test was shown to be below 2 - 4 hours. Therefore the results were based on the initially measured concentrations 54.7 - 111 - 230 - 610 - 790 µg dichlofluanid plus DMSA/L. The test concentration of 54.7 µg/L (total concentration), corresponding to 45.2 µg a.i./L (initially measured concentration of the active ingredient dichlofluanid) caused no mortality or non lethal effects. Therefore, the NOEC was laid down as 54.7 µg/L (total concentration), corresponding to 45.2 µg a.i./L (initially measured concentration of the active ingredient dichlofluanid). The LC_{50} after 96 h is 115 (99.9 - 132) µg/L (total concentration), corresponding to 90.1 (80.5 - 101) µg a.i./L (initially measured concentration of the active ingredient dichlofluanid).	
5.2.1	96h-LC_0	45.2 µg a.i./L	
5.2.2	96h-LC_{50}	90.1 µg a.i./L	X
5.2.3	96h-LC_{100}	436 µg a.i /L	
5.3	**Conclusion**	The validity criteria are summarised in Table A7_4_1_1-8. The test is considered valid.	
5.3.1	Other Conclusions	-	
5.3.2	Reliability	1	
5.3.3	Deficiencies	None	

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Annex Point IIA VII.7.1 *Cyprinodon variegatus* (Sheepshead minnow)

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	18/11/13
Materials and Methods	Applicant's version is acceptable noting the following: 1.1 – The year of publication of the study was 2004. This study is referred to by the UK CA as [redacted] (2004a).
Results and discussion	Applicant's version is acceptable noting the following: 4.2.2 – Measured concentrations of dichlofluanid were not maintained throughout the study. This result is to be expected given the rapid rate of hydrolysis of the active substance. Concentrations of the metabolite DMSA were also analytically determined. Calculating equivalent dichlofluanid concentrations from DMSA levels, total concentrations of dichlofluanid were maintained at > 80 % of nominal levels during the study. Measured concentrations of dichlofluanid only are presented in Table A.7.4.1.1-9 and study endpoints expressed in terms of mean measured concentrations of dichlofluanid are included in Table A.7.4.1.1-7. 5.2.2 – Based on mean measured concentrations of dichlofluanid only a LC_{50} of 10.54 µg a.s./L is derived at 24 h (the period during which all mortality occurred). It must be noted though that the fit of the dose response curve to the mean measured dichlofluanid data is visually poor and the reliability of the dose-response relationship is questionable.
Conclusion	Applicant's version is acceptable.
Reliability	1
Acceptability	Acceptable The study conforms to guideline OECD 203, was conducted to GLP and met all guideline validity criteria. The study is considered suitable for use in regulatory risk assessment.
Remarks	All endpoints and data presented in the summary and tables have been checked against the original summary and are correct. Given that continuous release is possible for PT21 products and since the metabolite DMSA may be less toxic to aquatic organisms than dichlofluanid, it is considered appropriate to use study endpoints based on mean measured concentrations of dichlofluanid only in the risk assessment. It is noted that this is potentially conservative as it assumes that the metabolite does not contribute to the toxicity seen in the study, i.e. that all effects are due to dichlofluanid exposure.
	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*

Section A7.4.1.1 Acute toxicity to fish

Annex Point IIA VII.7.1 *Cyprinodon variegatus* (Sheepshead minnow)

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: Dilution water

Criteria	Details
Source	ASPM seawater was diluted with demineralised water to a salinity of 21 ± 1.0 S. ASPM water is a defined seawater composed of reagent grade salts according to GUILLARD (1983). Chemical — Concentration [g/L] NaCl — 26.40 KCl — 0.84 $CaCl_2$ — 1.26 $MgCl_2 \cdot 6\ H_2O$ — 4.60 $MgSO_4 \cdot 7\ H_2O$ — 5.58 $NaHCO_3$ — 0.17 H_3BO_3 — 0.03
Alkalinity	Not measured
Hardness	Not measured
pH	See measured pH in Table A7_4_1_1-4
Oxygen content	>60 % saturation volume
Conductance	Instead, salinity was measured: 21.3 – 21.8 S during the test
Holding water different from dilution water	No

Table A7_4_1_1-2: Test organisms

Criteria	Details
Species/strain	*Cyprinodon variegatus* (Sheepshead minnow)
Source	Test fish were obtained from Aquatic Research Organisms, One Lafayette Road, Hampton, NH 03843-1271, USA. All fish used in the test originated from the same delivery of the supplier.
Wild caught	No; holding was performed at test facility in glass aquaria with filter system and ASPM water (acc. to GUILLARD 1983) at a salinity of 23 – 25 S, 22 ± 2 °C and diffuse light (0.1-10 µmol photons/m^2s, photoperiod 12 h daily with 15 to 30 min transition period). The water was changed at least once per week. The loading did not exceed 0.8 g (fresh weight) of fish/L. The dissolved oxygen concentration was more than 60 % of the air saturation value.
Age/size	Age not stated. The fish density in the tanks was less than 0.8 g fish per litre test solution. Average body length at the test start: 2.64 cm. Average body weight at the test start: 0.37 g.
Kind of food	Trouvit 40 / 2; MILKIVIT; D-86666 Bürgheim
Amount of food	The amount of food was 4 % of the fish body weight per feeding day.
Feeding frequency	Food was provided 3 times per week.
Pretreatment	Only Sheepshead minnow with at least 12 days of acclimatisation and mortality < 5 % during acclimatisation before the study starts were used in the test. During the final 48 h of acclimatisation fish were maintained in glass aquaria with background colours and light intensities similar to the test conditions. The test fish were not fed 48 h before test initiation.
Feeding of animals during test	No

Table A7_4_1_1-3: Test system

Criteria	Details
Test type	Static, performed in glass-aquaria loosely covered by glass tops
Renewal of test solution	No renewal of the test solution
Volume of test vessels	Volume not specified
Volume/animal	10 L/7 animals
Number of animals/vessel	7 animals/vessel
Number of vessels/ concentration	One vessel
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_1_1-4: Test conditions

Criteria	Details
Test temperature	22 ± 2 °C Control: 21.3 – 23.2 °C Test: 21.7 – 22.9 °C
Dissolved oxygen	90 – 100 % oxygen-saturation
pH	7.48 – 7.87
Adjustment of pH	No
Aeration of dilution water	Yes; gentle aeration was provided.
Intensity of irradiation	0.1 – 10 µmol/m^2 · s
Photoperiod	12 h light: 12 h dark, with 15 to 30 min transition period

Table A7_4_1_1-5: Dichlofluanid and DMSA (as equivalent of dichlofluanid) concentrations and recovery rates

Dilution level	Sampling date	Meas. Conc. of Dichlofluanid [µg a.i./L]	Calc. Conc. of Dichlofluanid [µg a.i./L]	Total Conc. [µg/L]	RR [%]
1:1	0 h	645**	145	790**	#
	2 h	803	573	1376	174**
	4 h	526	830	1356	172**
	24 h	—	—	n.a.	n.a.
	96 h	—	—	n.a.	n.a.
1:2	0 h	436	174	610	#
	2 h	363	319	682	112
	4 h	240	407	647	106
	24 h	—	—	n.a.	n.a.
	96 h	—	—	n.a.	n.a.
1:4	0 h	159	71.0	230	#
	2 h	139	179	318	138
	4 h	76.2	176	252	110
	24 h	< LOQ	264	264	115
	96 h	—	—	n.a.	n.a.
1:8	0 h	87.7	22.9	111	#
	2 h	63.9	50.1	114	103
	4 h	39.1	63.1	102	92
	24 h	1.18	105	106	95
	96 h	< LOQ	114	114	103
1:16	0 h	45.2	9.50	54.7	#
	2 h	20.0	32.5	52.5	96
	4 h	13.4	18.1**	31.5	58**
	24 h	4.50	46.8	51.3	94
	96 h	< LOQ	99.4**	99.4	182**

Meas. Conc. = Measured concentration (mean values of two injections)
Calc. Conc. = equivalent concentration of dichlofluanid calculated from the measured DMSA concentration
Total Conc. = Sum of the initially measured concentrations of the active ingredient dichlofluanid and its metabolite DMSA (expressed as dichlofluanid equivalents)
RR = Recovery rate based on the initially measured concentration (0 h)
– = Not analysed, due to 100 % mortality of the fish
n.a. = Not applicable
# = 0 h values were set to 100 % for the evaluation of the RR after 2, 4, 24 and 96 h
** = reanalysed twice, were not taken into account. These concentrations did not fit with the dilution level and the degradation kinetics.

Table A7_4_1_1-6: Cumulative mortality and behavioural observations

Total Concentration [µg/L]	Exposure time									
	6 h		24 h		48 h		72 h		96 h	
	Dead	Obs.	Dead	Obs.	Dead	Obs.	Dead	Obs.	Dead	Obs.
790	100	7/7**(E)	100	-	100	-	100	-	100	-
610	100	7/7**(E)	100	-	100	-	100	-	100	-
230	0	7/7 (2.1)	100	7/7 (E)	100	-	100	-	100	-
111	0	5.7 (1) 2/7 (2.1)	43	1/7 (2.1) 3/7 (2.1+4.1) 3/7 (E)	43	3/4 (2.4) 1/4 (4.1)	43	3/4 (2.4) 1/4 (4.1)	43	1/4 (1) 3/4 (2.5)
54.7	0	7.7 (1)	0	7.7 (1)	0	7.7 (1)	0	7.7 (1)	0	7.7 (1)
Control	0	7.7 (1)	0	7.7 (1)	0	7.7 (1)	0	7.7 (1)	0	7.7 (1)

The numbers in brackets correspond to the following observations:
(1) = Normal behaviour (E) = Exitus lethalis
(2.1) = Lethargy (2.5) = Missing escape reflex
(2.4) = Slow escape reflex (4.1) = Hyperventilation
**Fish were already dead after 4 h

Table A7_4_1_1-7: LC-Values with 95 % Confidence Intervals

LC_{50} –values (CI 95 %)			
Test duration	**Total concentration [µg/L]**	**Initially measured concentration of the a.i. dichlofluanid [µg a.i./L]**	**Mean measured concentration of dichlofluanid [µg a.i./L]**
6 h	375 (340 – 413)	263 (238 – 291)	42.63
24 h	115 (99.9 – 132)	90.1 (80.5 – 101)	10.54
48 h	115 (99.9 – 132)	90.1 (80.5 – 101)	-
72 h	115 (99.9 – 132)	90.1 (80.5 – 101)	-
96 h	115 (99.9 – 132)	90.1 (80.5 – 101)	-
LC_{100}-, LC_0- and NOEC-values			
	Total concentration [µg/L]	**Initially measured concentration of the a.i. dichlofluanid [µg a.i./L]**	**Mean measured concentration of dichlofluanid [µg a.i./L]**
LC_{100} Lowest test item concentration with 100 % mortality after 96 h	610	436	10.17*
LC_0 Highest test item concentration with 0 % mortality after 96 h	54.7	45.2	9.25*
NOEC (0 – 96h) No observed effect concentration	54.7	45.2	-
LC_{10}	-	-	2.47

a.i. = active ingredient of the test item (dichlofluanid)
Total concentration = sum of the initially measured concentrations of the active ingredient dichlofluanid and its metabolite DMSA (expressed as dichlofluanid equivalents

* Value determined after 24 h

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%	**X**	
Concentration of dissolved oxygen in all test vessels > 60% saturation	**X**	
Concentration of test substance ≥ 80% of initial concentration during test (otherwise determination of effect levels based on mean measured concentrations)	**X**	

Criteria for poorly soluble test substances	**X**	

Table A7_4_1_1-9: Measured concentrations of dichlofluanid in water (µg/L)

Dilution level	0 h	2 h	4 h	24 h	96 h	Mean measured
1:16	45.2	20	13.4	4.5	<LOQ	9.25[1]
1:8	87.7	63.9	39.1	1.18	<LOQ	9.8[1]
1:4	159	139	76.2	<LOQ	NA	10.17[1]
1:2	436	363	240	NA	NA	342.67[2]
1:1	645	803	526	NA	NA	683.9[2]

NA = Not analysed
[1] Averaged over 24 h period since all mortality occurred within 24 h
[2] Averaged over 4 h period since all mortality occurred within 4 h

Figure A7-4-1-1-1: Concentration-effect curve for surivial over 24 h period based on mean measured concentrations

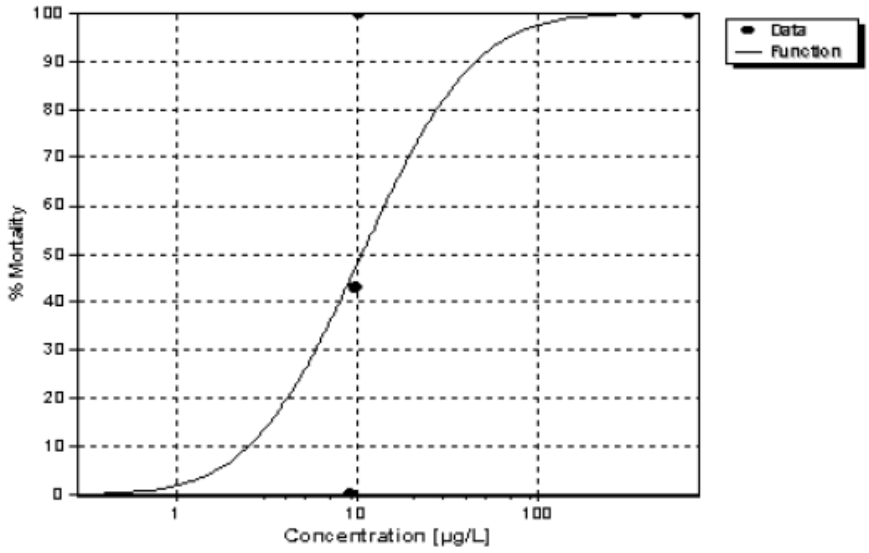

Fig. 3: Concentration-effect curve showing the influence of the test item on survival of the introduced Sheepshead Minnow as observed after 24 h