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The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides and Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/ehs/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organisations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. UNDP is an observer. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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FOREWORD

This document presents **Part 1** of the report of Phase 2 of the validation of the Zebrafish Embryo Toxicity Test. The project to develop a Zebrafish Embryo Toxicity Test was proposed by Germany to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in 2004. The European Centre for the Validation of Alternative Methods (ECVAM) was charged with the validation studies. The report of Phase 1 of the validation was published in 2011 (No. 157 in the Series on Testing and Assessment).

The Phase 2 validation report was prepared by a Validation Management Group, coordinated by ECVAM. The aim of Phase 2 was to further evaluate the intra- and inter-laboratory reproducibility of the test with thirteen chemicals covering a wide range of physical-chemical properties, uses and modes of action.

The **Part 2**, provided in a separate document includes 10 annexes:

Annex I	Phase 2 Documents and Method Description
Annex II	Selection of Chemicals for Phase 2
Annex IIIa	Analysis of Three Chemicals in Fish Embryo Test Stock and Exposure Solutions for Phase 2b by P&G
Annex IIIb	Analysis of Two chemicals in Fish Embryo Test Stock and Exposure Solutions for Phase 2b by Ipo-Pszczyna
Annex IV	Overview of Runs
Annex V	Statistical Report Phase 2
Annex VI	Standard Operation Procedure - Zebrafish Embryo Toxicity Test (SOP ZFET OECD V02.10)
Annex VIIa	Trial plan Phase 2a – Training of new laboratories (TP_ZFET_OECD_2a V01)
Annex VIIb	Trial plan Phase 2b – Testing of 13 chemicals (TP_ZFET_OECD_2b V01_1)
Annex VIIc	Amendment to trial plan Phase 2b (TP_ZFET_OECD_2b V01_1 amendment)
Annex VIII	Evaluation of Time-Dependent Changes in LC50s during the Zebrafish Fish EmbryoTest Using Data Gathered from Phase 1 and 2 of the OECD Validation of the Zebrafish FET
Annex IX	Evaluation of Hatching during the Zebrafish Fish Embryo Test Using Data Gathered from Phase 2 of the OECD Validation of the Zebrafish FET
Annex X	Impact of the group size on the estimation of LC50 in the Zebrafish Fish Embryo Test

The report was reviewed, commented on, and approved by the OECD *Ad hoc* Expert Group on the Fish Embryo Toxicity Test, which met on 16-17 February 2012 in Berlin, Germany. The report was subsequently endorsed by the WNT in April 2012. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (hereafter Joint Meeting) agreed to its declassification on 26 July 2012. This document is published under the responsibility of the Joint Meeting.

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SUMMARY

In autumn 2005, the German Federal Environment Agency submitted the draft Test Guideline (TG) "Fish embryo toxicity (FET) test" to the OECD Test Guideline Programme together with a supportive Background Paper. Subsequently, OECD established the *adhoc* Expert Group on the Fish Embryo Toxicity Test. Based on the outcome of two expert meetings, OECD decided to perform a validation study (coordinated by ECVAM and steered by a validation management group, VMG).

The study was divided into two phases: The aim of Phase 1 was to evaluate the transferability, and the intra- and inter-laboratory reproducibility of the zebrafish FET (ZFET) with seven chemicals. The VMG concluded that the ZFET test was successfully transferred from the lead laboratory to the participating laboratories. The report of Phase 1 was published in 2011 on the OECD website (OECD 2011).

The aim of Phase 2 was to further evaluate the intra- and inter-laboratory reproducibility of the ZFET with an additional thirteen chemicals covering specific areas of use (chemicals, pharmaceuticals, pesticides, biocides), a wide range of toxicity and various modes of action.

The chemicals were tested at five different concentrations in three independent runs in four laboratories (except for methylmercury (II) chloride which was assessed in three laboratories) with appropriate controls. Stock solutions and test concentrations were analytically confirmed for carbamazepine, prochloraz, 1-octanol, copper (II) sulfate pentahydrate and tetradecyl sulfate sodium salt.

In brief, newly fertilized zebrafish eggs were exposed for 96h to the chemicals. Up to four apical endpoints were recorded daily as indicators of acute lethality in fish: coagulation of the egg, lack of somite formation, non-detachment of the tail bud from the yolk sac and lack of heart-beat. LC50 values were calculated for 48h and 96h exposure time points.

In general, the results of Phase 2 confirm the findings of Phase 1. The ZFET was successfully transferred to four new laboratories participating in Phase 2. For nine chemicals, the intra- and inter-laboratory reproducibility of the ZFET is acceptable with coefficients of variation (CV) below 30% regardless of the chemical or the laboratory. For three chemicals (Merquat 100, methylmercury (II) chloride, copper (II) sulfate pentahydrate) CVs >30% were calculated. However, a factor contributing to the large CVs is the very high acute toxicity of these three chemicals, since relatively small differences in the LC50 values are magnified and result in a larger CV. With prochloraz tested close to its limit of solubility acceptable intra- and inter-laboratory reproducibility was achieved only in two laboratories. As expected the chorion acts as a barrier for chemicals with high molecular weight, i.e. for the two polymers tested with the ZFET (Merquat 100 and Luviquat HM 552) some lethality was observed at 48h and LC50s were mostly confined to 96h exposures (roughly 24h post-hatch).

It was not possible to find a time-dependant pattern of toxicity for chemical categories other than the above mentioned cationic polymers. Evaluation of the effect of the group size/concentration confirms the use of 20 embryos/group. The hatching rate in the negative control was consistent and it might be useful to include into the ZFET acceptance criteria that hatching in the negative control should exceed 80% at 96h.

For the 13 chemicals tested in Phase 2, the predictive capacity of ZFET for acute fish toxicity is very promising but will need to be underpinned with additional data.

INTRODUCTION

In autumn 2005, the German Federal Environment Agency (UBA) submitted the draft Test Guideline (TG) "Fish embryo toxicity (FET) test" to the OECD Test Guideline Programme (Project 2.7) together with a supportive Background Paper (Braunbeck *et al.*, 2005). Based on the comments received from the national coordinators, the OECD decided to establish the *ad hoc* Expert Group on the Fish Embryo Toxicity Test. During several teleconferences and face-to-face meetings, the submitted documents were reviewed taking into consideration the scientific basis, reproducibility and predictive capacity of the FET. A thorough re-evaluation of existing data demonstrated that the FET correlates well with acute fish toxicity tests (Lammer *et al.*, 2009). The *ad hoc* Expert Group noted that most data were available for the zebrafish embryo toxicity test (ZFET), however, data providing sufficient evidence for the reproducibility of the method were lacking.

In May 2008, OECD asked the European Centre for the Validation of Alternative Methods (ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Italy) to coordinate the "ZFET Performance Study". A Validation Management Group (VMG) was established in November 2008. After further discussions, the VMG agreed that the study would be divided into two phases, where Phase 1 constitutes the transferability of the ZFET from the lead laboratory to the other laboratories (Phase 1a) and subsequently the testing of six chemicals (Phase 1b). In Phase 2, 13 chemicals would be tested.

As agreed upon by the VMG and the OECD *ad hoc* Expert Group on Fish Embryo Tests, new laboratories joining the study for Phase 2 would need to undergo training (Phase 2a). This training step is based on the trial plan used for Phase 1a (see Annex I). The Phase 2a study was conducted from October 2010 to February 2011. 3,4−Dichloroaniline (3,4-DCA) was used as a positive control test chemical since it is well established as described in the Fish Egg Toxicity test for waste water testing (DIN, 2001). The four new participating laboratories transferred the Standard Operation Procedure (SOP) by testing three independent runs of 3,4-DCA using five test concentrations.

The Phase 2b study was conducted from February 2011 to November 2011. Nine laboratories trained in Phases 1a or 2a tested thirteen chemicals in three independent runs. 3,4-DCA was used as positive control at a concentration of 4.0 mg/L (for detailed study design see Section 8.1).

VALIDATION MANAGEMENT GROUP (VMG)

The VMG steers the study and is responsible for the overall study design. Specific roles and responsibilities are listed below:

Name	Affiliation	Role
Marlies Halder François Busquet (until January 2012)	JRC/IHCP/ECVAM Ispra, ITALY	Coordination/reporting
Patric Amcoff (until April 2011) Anne Gourmelon	OECD Environment, Health and Safety Division, Environment Directorate Paris, FRANCE	OECD Test Guideline Programme
Thomas Braunbeck	University of Heidelberg Heidelberg, GERMANY	Lead laboratory & German Federal Environment Agency (Umweltbundesamt; UBA) representative (until April 2010)
Scott Belanger	Procter & Gamble Cincinnati, OH, USA	Participating laboratory
Greg Carr	Procter & Gamble Cincinnati, OH, USA	Data analysis for Phase 1b and Phase 2
Adam Lillicrap	NIVA Oslo, NORWAY	Independent adviser
Susanne Walter-Rohde	German Federal Environment Agency	Lead country OECD project 2.7 (joined the VMG in

	(Umweltbundesamt ; UBA), Dessau-Roßlau, GERMANY	April 2010)

PARTICIPATING LABORATORIES

Laboratory	Responsible
University of Heidelberg, Heidelberg, GERMANY[1]	Thomas Braunbeck
Procter & Gamble, Cincinnati, OH, USA[2]	Scott Belanger
Ipo-Pszczyna, Pszczyna, POLAND[2]	Przemysław Fochtman
Merck KGaA, Darmstadt, GERMANY[3]	Nicole Huebler
UBA, Berlin, GERMANY[3]	Carola Kussatz, Christian Polleichtner
IVM, Amsterdam, THE NETHERLANDS	Juliette Legler
Escuela Nacional de Ciencias Biológicas, IPN, México City, MEXICO[3]	Fernando Martínez-Jerónimo
BASF, Ludwigshafen, GERMANY[3]	Edward Salinas
RIVM, Bilthoven, THE NETHERLANDS	Leo van der Ven

[1] Lead laboratory

[2] Performed analytical measurements.

[3] New laboratories

Note: Two laboratories involved in Phase 1 did not participate in Phase 2.

DEFINITION OF THE SOP

Taking into consideration the concerns expressed by the *ad hoc* Expert Group on the OECD draft guideline (OECD, 2006) the SOP covers the following points:

- The exposure duration was extended beyond hatch to 96h and calculation of LC50 at 48h and 96h, since the chorion could act as a barrier to chemical exposure of the embryo.
- The number of embryos per concentration and control was increased to 20 embryos instead of 10 embryos.
- Acceptance criteria were set for the fertilisation rate, for the negative internal control, and modified for the positive control.

CHEMICALS AND TEST CONCENTRATIONS

Chemicals were selected based on the recommendations of the *ad hoc* Expert Group (see minutes of the FET II Expert consultation meeting, May 2008). Since the chemical selection is a critical step for a validation study and it is meant to define the applicability domain of the test method, the VMG agreed to reactivate the Chemical Selection Group (CSG) established at the 1st meeting of the OECD FET Expert Consultation Meeting (FET I ECM; 9-11 October 2007, Berlin).

An extended list of 20 chemicals developed by the CSG and agreed by the VMG was presented to the OECD FET *ad hoc* expert group during a teleconference call on 30th June 2010. Thirteen chemicals were selected as chemicals to be tested in Phase 2. The rationale behind the chemicals selection is described in detail in Annex II together with their properties, modes of action, areas of use etc.

The lead laboratory (University of Heidelberg) and one participating laboratory (Procter & Gamble, P&G) performed the range-finding tests for Phase 2b. Since it was not possible to determine an LC50 value for n-butylamine, morpholine, ivermectin and dieldrin, the VMG decided to test chemicals with similar properties and toxicity to fish (Annex II).

It is acknowledged that some of the chemicals for Phase 2 were sponsored by participating laboratories:

- dimethyl sulfoxide by the University of Heidelberg;
- Luviquat HM 552 and triethylene glycol by BASF;
- 2,4-dinitrophenol by Merck KGaA;
- carbamazepine and methylmercury (II) chloride by UBA;
- tetradecyl sulfate sodium salt by P&G and
- the 6 remaining chemicals by ECVAM.

ECVAM aliquoted and distributed the 13 chemicals to the participating laboratories (see Table 3 in section 8) including the Material Safety Data Sheets and Lot Certificate of Analysis. The chemicals were not coded as agreed upon by the OECD FET *ad hoc* expert group during a teleconference call on 30th June 2010. The same lot of 3,4-DCA (positive control) was used for the whole study (Phase 1 and Phase 2).

Table 1 lists the test chemicals and concentrations tested. The preparation of the stock solutions and test concentrations is given in the trial plan (Annex VII).

Table 1: Phase 2b chemicals: physical chemical properties and test concentrations (see also Annex II)

Chemical	Fish Toxicity	CAS Number	Catalogue Number	Lot Number	MW (g/mol)	Log Kow	HLC (Pas-m3/mole)	Test Concentrations (mg/L)
Methylmercury (II) chloride	+++	115-09-3	33368	szba172x	251.08	0.41[db]	NA	0.00625, 0.0125, 0.025, 0.05, 0.1
Copper (II) sulfate pentahydrate	+++	7758-99-8	209198	mkbd0338	249.68	NA	NA	0.15, 0.3, 0.6, 1.2, 2.4
4,6-Dinitro-*o*-cresol	+++	534-52-1	45464	sze6159x	198.14	2.13[db]	1.4E-06[db]	0.18, 0.32, 0.58, 1.05, 1.89
2,4-Dinitrophenol	+++	51-28-5	34334	sze9167x	184.11	1.67[db]	8.06E-08[db]	0.625, 1.25, 2.5, 5, 10
Merquat 100	++	26062-79-3	409022	mkbf20418v	200,000-350,000	-2.49[est]	7.2E-12[est]	0.1. 0.2, 0.4, 0.8, 1.6
Luviquat HM 552	++	95144-24-4	59059	1322472	~400,000	1.38[est]	1.87E-14[est]	0.125, 0.25, 0.5, 1, 2
Tetradecyl sulfate sodium salt	++	1191-50-0	293938	0600LC	316.43	2.67[est]	3.25E-07[est]	0.156, 0.3125, 0.625, 1.25, 2.5
Malathion	++	121-75-5	PS86	447-115b	330.4	2.36[db]	8.39E-10[est]	0.5, 1, 2, 4, 8
Prochloraz	++	67747-09-5	45631	sze6220x	376.67	4.1[db]	7.58E-12[est]	0.5, 1, 2, 4, 8

1-Octanol	+	111-87-5	293245	stbb5181	130.23	3.00[db]	2.45E-05[db]	2.5, 5, 10, 20, 40
Carbamazepine	+	298-46-4	C4024	119k1317v	236.28	2.45[db]	1.08E-10[est]	54.7, 76.5, 107.1, 150, 210
Dimethyl sulfoxide	-	67-68-5	10282	0215	78.13	-1.35[db]	4.96 E-08[est]	10, 17, 28.9, 49.13, 83.521
Triethylene glycol	-	112-27-6	T59455	stbb7542	150.17	-1.75[est]	3.16E-011[est]	20, 30, 45, 67.5, 101.25

- = non-toxic (LC50>100 mg/L); + = moderately toxic (LC50=10-100 mg/L); ++ = toxic (LC50=1-10 mg/L; +++ = very toxic (LC50<1 mg/L);
NA = Not available; MW = Molecular Weight; HLC = Henry's Law Constant; db = experimental database match; est = estimated;
Note: log Kow and HLC were estimated using EPISUITE 4.0 (2008) except when measured values were available (cited within EPISUITE).
All chemicals were purchased from Sigma-Aldrich except for dimethyl sulfoxide (Gruessing GmbH)

PHASE 2A – TRANSFER OF THE SOP

Study design

Before the start of the training, the SOP was distributed to the four new laboratories and discussed. The four laboratories assessed the transferability of the SOP by testing 3,4-DCA in five concentrations (0.5, 1, 2, 4, and 8 mg/L plus negative control). For further details see Annex I.

For each test, measurements of test conditions such as dissolved oxygen concentration, pH, total hardness, temperature and conductivity were performed for the controls and the highest concentration as described in the respective SOP.

In contrast to Phase 1, the concentration of the stock solutions were not confirmed by analytical measurements; however, the participating laboratories were asked to store samples.

LC50 values were calculated for 48h and 96h exposure following the recommendations of the OECD Guidance Document 54 on the statistical analysis of ecotoxicity data (OECD, 2006). Details on statistical analysis and software used are given in Annex V).

With regard to intra- and inter-laboratory reproducibility, the VMG agreed upon that coefficients of variation (CV) below 30% would be acceptable for demonstration of the transferability of the SOP using 3,4-DCA.

Results LC50 values - Three runs with 3,4-DCA

The laboratories provided the data to ECVAM using the corresponding reporting templates (see Annex I). Prior to statistical analysis by P&G, the data underwent a quality check, i.e. it was checked whether complete information was provided and whether the runs met the acceptance criteria as described in the SOP.

One run of laboratory J, had to be repeated since it did not meet one of the acceptance criteria (fertility rate <70%). The other runs of the laboratories met the acceptance criteria as defined in the SOP.

The LC50 values of the three independent runs per laboratory are given in Table 2 (the detailed report of the statistical analysis is available in Annex V). Table 2 shows the intra- and inter-laboratory reproducibility of the LC50 values.

Table 2: Three runs with 3,4-DCA: Combined LC50 values and intra-laboratory and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

3,4-DCA	**Combined LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory H	3.86	2.65	5.47	5.50
Laboratory I	2.81	2.55	12.28	17.72
Laboratory J	3.41	2.70	34.09	19.03
Laboratory K	3.79	3.20	10.08	22.42
			Inter-laboratory CV (%)	
All laboratories (Phase 1a & 2a)	3.47	2.77	*27.4*	*26.4*

CV: coefficient of variation

Conclusions Phase 2a

The VMG concluded that the ZFET could be successfully transferred from the lead laboratory to the four new participating laboratories.

PHASE 2B – TESTING OF THIRTEEN CHEMICALS

Study design

As described for Phase 1, the nine laboratories were asked to test the chemicals in three independent runs using the pre-defined test concentrations (see Table 1). For each run, measurements of test conditions such as dissolved oxygen concentration, pH, total hardness, light intensity, temperature and conductivity were performed for the controls and the highest concentration as described in the SOP (Annex I, Annex VI).

The results of Phase 1b resulted in the following amendment to the SOP:

- A note on acceptance criteria for internal negative controls was added: "If more than 1 dead embryo is observed in the internal negative control, the plate might be rejected."

P&G carried out the analytical measurement of the three chemicals (1-octanol, tetradecyl sulfate sodium salt, copper (II) sulfate pentahydrate) tested in their laboratory by measuring the stock solutions and the test concentrations of one run per chemical (see Annex IIIa for full report).

Ipo-Pszczyna performed the analytical measurement of two chemicals (carbamazepine, prochloraz) tested in their laboratory by measuring the stock solutions and the test concentrations of the three runs per chemical (see Annex IIIb for full report).

All the laboratories were asked to store samples of the stock solutions of the chemicals, since it might be necessary to confirm their concentration.

LC50 values were calculated for 48h and 96h exposure following the recommendations of the OECD Guidance 54 in the statistical analysis of ecotoxicity data (OECD, 2006). Details on statistical analysis and software used are given in Annex V.

With regard to intra- and inter-laboratory reproducibility, the VMG agreed that coefficients of variation (CV) below 30% would be acceptable. However, this should be regarded as an indicative value since for difficult chemicals CV >30% can be expected.

Since not all laboratories had the capacity to test all chemicals, the VMG decided to distribute the 13 chemicals amongst the laboratories as given in Table 3. Thus, it could be ensured that the laboratories tested chemicals with different range of toxicities and each chemical was tested in four laboratories. It should be noted that only three laboratories could test methylmercury (II) chloride.

Table 3: Distribution of chemicals over the nine laboratories

		Laboratories								
Chemicals	**Fish Toxicity**	**B**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
Methylmercury (II) chloride[1, 2]	+++		X		X				X	
Copper (II) sulfate pentahydrate [1]	+++				X	X[3]	X	X		
4,6-Dinitro-*o*-cresol	+++			X	X		X			X
2,4-Dinitrophenol	+++		X		X			X		X
Merquat 100	++				X		X		X	X
Luviquat HM 552	++				X		X	X		X
Tetradecyl sulfate sodium salt [1]	++	X			X	X[3]	X			
Malathion	++	X			X				X	X
Prochloraz	++			X[3]	X		X	X		
1-Octanol	+	X			X	X[3]	X			
Carbamazepine	+		X	X[3]	X					X
Dimethyl sulfoxide	-				X		X		X	X
Triethylene glycol	-	X		X	X			X		

1) Methylmercury (II) chloride; copper (II) sulfate pentahydrate and tetradecyl sulfate sodium salt are listed according to the fish toxicity of their soluble form.
2) Tested only in three laboratories; due to safety reasons no further laboratory could test the chemical.
3) Analytical measurements of stock solutions and test concentrations were carried out. Reports on the analytics are available in Annexes IIIa and IIIb.

Results

The laboratories provided data of 153 runs to ECVAM using the reporting template (see Annex I). Prior to statistical analysis by P&G, the data underwent a quality check.

Out of the 153 runs, 10 runs did not meet the acceptance criteria and were disqualified for the following reasons: five runs due to increased lethality in the negative external control, three runs due to increased lethality in the negative internal control, one run due to reduced lethality in the positive control and one run due to the low fertility rate of the eggs.

The disqualified runs were reported by the laboratories as follows:

- Two runs of laboratory B (malathion and 1-octanol) due to increased lethality (>10%) in the negative external control.
- Two runs of laboratory D (carbamazepine and 2,4-dinitrophenol) did not meet the acceptance criteria since the lethality in the negative external control was >10%.
- Three runs of laboratory E did not meet the acceptance criteria. For one run (carbamazepine) the lethality in the positive control was <30% and for two runs (4,6-dinitro-*o*-cresol and triethylene glycol), the lethality in the negative internal control was too high.
- One run of laboratory I (prochloraz) did not meet the acceptance criteria since the lethality in the negative internal control was too high.
- Two runs of laboratory J did not meet the acceptance criteria. For one run (methylmercury (II) chloride) the lethality in the negative external control was >10%, and for the other run (malathion) the fertility rate was <70%.

The laboratories were asked to repeat the disqualified runs and all of the repeated runs met the acceptance criteria.

For a complete overview on the disqualified runs of Phase 1 and Phase 2, see Annex IV.

NOTE: In the following sections, the mean LC50 values and the intra- and inter-laboratory reproducibility are given for the individual chemicals. The LC50 values for each run are available in the full statistics report in Annex V. The LC50 values were calculated based on the nominal concentrations.

Methylmercury (II) chloride

The mean LC50 values of the three independent runs per laboratory are given in Table 4.

Table 4: Methylmercury (II) chloride (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Methylmercury (II) chloride*	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory D	0.0213	0.0131	5.11	11.15
Laboratory F	0.0606	0.0411	0.00	3.53
Laboratory J	0.0443	0.0299	3.73	2.14
			Inter-laboratory CV (%)	
All laboratories	0.0421	0.0280	46.9	50.19

*only three laboratories were able to test the chemical (see Table 3); CV: coefficient of variation

- The intra-laboratory reproducibility at 48h and 96h is acceptable in all laboratories.
- The inter-laboratory reproducibility at 48h (CV = 46.9%) and 96h (CV = 50.19%) is not acceptable with regard to the CV <30% criteria. However, a factor contributing to the large CVs is the very high acute toxicity of the chemical, since small differences in the LC50 values are magnified resulting in a larger CV.
- Comparison of the mean LC50 values at 48h and 96h indicate an increase in toxicity by factor 1.5 at 96h.

Copper (II) sulfate pentahydrate

Analysis of Copper (II) sulfate pentahydrate stock solutions and test concentrations

- P&G performed analytical measurements of the copper (II) sulfate pentahydrate stock solutions and of the test concentrations for run 2 (for details see Annex IIIa).
- The average of the stock solution samples from the three independent runs was 72.9% of the nominal concentration.
- Geometric mean measured concentrations throughout the test were 59.4-64.8% of the nominal concentrations. Minimal losses over the 24h renewal period were observed across all concentrations.

LC50 values – Copper (II) sulfate pentahydrate

The mean LC50 values of the three independent runs per laboratory are given in Table 5.

Table 5: Copper (II) sulfate pentahydrate (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Copper (II) sulfate pentahydrate	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory F	0.198	0.198	14.91	14.91
Laboratory G	0.302	0.302	12.89	12.89
Laboratory H	0.243	0.241	9.91	9.20
Laboratory I	0.491	0.423	16.45	7.24
			Inter-laboratory CV (%)	
All laboratories	0.308	0.291	41.72	33.63

CV: coefficient of variation

- The intra-laboratory reproducibility at 48h and 96h is acceptable.
- With regard to the CV <30% criteria, the inter-laboratory reproducibility is not acceptable at 48h (CV = 41.72%) and, although better, at 96h (CV = 33.63%). As methylmercury (II) chloride, copper (II) sulfate pentahydrate has a relatively high toxicity and similar statistical considerations apply (see 8.2.1).
- There is no difference in toxicity at 48h and 96h.

4,6-Dinitro-o-cresol

The mean LC50 values of the three independent runs per laboratory are given in Table 6.

Table 6: 4,6-Dinitro-o-cresol (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

4,6-Dinitro-*o*-cresol	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory E	0.728	0.597	30.86	1.68
Laboratory F	0.749	0.509	27.84	14.28
Laboratory H	0.704	0.601	9.65	12.44
Laboratory K	0.710	0.561	26.49	18.52
			Inter-laboratory CV (%)	
All laboratories	0.723	0.567	2.79	7.52

CV: coefficient of variation

- The intra-laboratory reproducibility at 48h and 96h is acceptable apart from laboratory E (CV = 30.86%) at 48h. The intra-laboratory reproducibility at 96h is lower than at 48h in three laboratories and higher in one laboratory.
- The inter-laboratory reproducibility at 48h and 96h is <10%.
- Comparison of the mean LC50 values at 48h and 96h indicate a slight increase in toxicity at 96h.

2,4-Dinitrophenol

The mean LC50 values of the three independent runs per laboratory are given in Table 7.

Table 7: 2,4-Dinitrophenol (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

2,4-Dinitrophenol	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory D	3.04	2.71	20.58	32.72
Laboratory F	5.07	4.01	1.15	16.78
Laboratory I	3.59	2.59	23.60	1.56
Laboratory K	4.81	2.67	8.39	5.28
			Inter-laboratory CV (%)	
All laboratories	4.13	3.00	23.54	22.66

CV: coefficient of variation

- The intra-laboratory reproducibility at 48h and 96h is acceptable except for laboratory D (CV = 32.76%) at 96h.
- The inter-laboratory reproducibility at 48h and 96h is acceptable (CVs <25%).
- Comparison of the mean LC50 values at 48h and 96h indicate an increase in toxicity by factor 1.5 at 96h.

Merquat 100

The mean LC50 values of the three independent runs per laboratory are given in Table 8.

Table 8: Merquat 100 (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Merquat 100	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory F	A	0.522	NA	17.13
Laboratory H	1.360*	0.321	NA	26.68
Laboratory J	A	0.753	NA	37.21
Laboratory K	A	0.415	NA	1.91
			Inter-laboratory CV (%)	
All laboratories	AA	0.496	NA	40.84

A: not possible to calculate a reliable mean LC50 due to the absence of lethality in the highest concentrations of the 3 runs; AA: not possible to calculate; NA: CV could not be calculated; *LC50 from one run; CV: coefficient of variation

- At 48h, the intra- and inter-laboratory reproducibility could not be calculated since there was insufficient lethality observed (except for one run of laboratory H) to derive a LC50.
- The intra-laboratory reproducibility at 96h is acceptable except for laboratory J (CV = 37.21%).
- The inter-laboratory reproducibility at 96h is not acceptable (CV = 40.84%) when applying the CV <30% criteria. However, a factor contributing to the large CV is the very high acute toxicity of the chemical. Small differences in the LC50 values are magnified resulting in a larger CV as already seen for copper (II) sulfate pentahydrate and methylmercury (II) chloride.
- The enhanced post-hatch mortality indicates that insufficient amounts of the polymer Merquat 100 passed the chorion to cause consistent toxicity due to molecular weight (200,000 – 350,000 g/mol). In fact, Merquat 100 had been selected to challenge the barrier function of the chorion.

Luviquat HM 552

The mean LC50 values of the three independent runs per laboratory are given in Table 9.

Table 9: Luviquat HM 552 (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Luviquat HM 552	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory F	A	0.826	NA	7.76
Laboratory H	A	0.744	NA	11.55
Laboratory I	A	1.200	NA	13.46
Laboratory K	1.450*	0.738	NA	9.13
			Inter-laboratory CV (%)	
All laboratories	AA	0.876	NA	24.77

A: not possible to calculate a reliable mean LC50 due to the absence of lethality in the highest concentrations of the 3 runs; AA: not possible to calculate; NA: CV could not be calculated; *LC50 from one run; CV: coefficient of variation

- At 48h, the intra- and inter-laboratory reproducibility could not be calculated since there was insufficient lethality observed (except for one run of laboratory K) to derive a LC50.
- The intra-laboratory reproducibility at 96h is acceptable (CV <15%).
- The inter-laboratory reproducibility at 96h is acceptable (CV = 24.77%).
- As for Merquat 100, the enhanced post-hatch lethality indicates that insufficient amounts of the polymer Luviquat HM 552 passed the chorion to cause consistent toxicity. Luviquat HM 552 had also been selected to challenge the barrier function of the chorion.

Tetradecyl sulfate sodium salt

Analysis of tetradecyl sulfate sodium salt stock solutions and test concentrations

- P&G performed analytical measurements of the tetradecyl sulfate sodium salt stock solutions and of the test concentrations for run 2 (for details see Annex IIIa).
- The average of the stock solution samples from the three independent runs was 112.5% of nominal concentration indicating that the stock solution was accurately prepared.
- Final measured test concentrations were calculated as the arithmetic mean of the arithmetic means for each time-point. The loss of alkyl sulfates, based on historical knowledge, is known to occur rapidly; therefore, geometric means were not used in this instance to calculate measured test concentrations.
- Measured test concentrations were 55.1-114.2% of nominal. Substantial losses over the 24h renewal period were observed across all concentrations.

LC50 values – Tetradecyl sulfate sodium salt

The mean LC50 values of the three independent runs per laboratory are given in Table 10.

Table 10: Tetradecyl sulfate sodium salt (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Tetradecyl sulfate sodium salt	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory B	0.385	0.381	35.67	34.02
Laboratory F	0.304	0.304	4.45	4.45
Laboratory G	0.424	0.435	32.83	28.17
Laboratory H	0.236	0.236	15.88	15.88
			Inter-laboratory CV (%)	
All laboratories	0.337	0.339	24.99	25.79

CV: coefficient of variation

- The intra-laboratory reproducibility at 48h and 96h is acceptable except for laboratory B at 48h (CV = 35.67%) and 96h (CV = 34.02%) and for laboratory G at 48h (CV = 32.83%).
- The inter-laboratory reproducibility is acceptable at 48h and 96h (CVs <26%).
- Comparison of the mean LC50 values at 48h and 96h indicates no increase in toxicity.

Malathion

The mean LC50 values of the three independent runs per laboratory are given in Table 11.

Table 11: Malathion (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Malathion	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory B	7.77*	4.97**	NA	19.04
Laboratory F	4.61	3.69	14.39	8.80
Laboratory J	A	4.87	NA	10.28
Laboratory K	5.99**	4.71	18.68	33.19
			Inter-laboratory CV (%)	
All laboratories	6.12	4.56	25.83	12.98

A: not possible to calculate a reliable mean LC50 due to the absence of lethality in the highest concentrations of the 3 runs; NA: CV could not be calculated; *LC50 from one run; **mean LC50 from two runs; CV: coefficient of variation

- At 48h, the intra-laboratory reproducibility of laboratories F and K was acceptable (CVs <20%), whereas it could not be calculated for laboratories B and J, since there was insufficient lethality observed (except for one run of laboratory B) to derive a LC50.
- At 96h, the intra-laboratory reproducibility is acceptable (CVs <20%) for all the laboratories except for laboratory K (CV = 33.19%).
- The inter-laboratory reproducibility is acceptable at 48h (CV = 25.83%) and two-times lower at 96h (CV = 12.98%).
- The mean LC50 values indicate that malathion is slightly more toxic at 96h.

Prochloraz

Analysis of prochloraz stock solutions and test concentrations

- Ipo-Pszczyna performed analytical measurements of the prochloraz stock solutions and of the test concentrations for each run (for details see Annex IIIb).
- For run 1 and run 3, the measured stock solution concentration as well as the test concentrations proved that prochloraz has been satisfactorily maintained throughout the test (>80% of the nominal concentration).
- For run 2, the measured stock solution and test concentrations were not maintained throughout the test.

LC50 values – Prochloraz

The mean LC50 values of the three independent runs per laboratory are given in Table 12.

Table 12: Prochloraz (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Prochloraz	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory E	A	7.87*	NA	NA
Laboratory F	4.65	4.62	11.79	11.31
Laboratory H	4.28	4.02	8.89	8.74
Laboratory I	A	5.90*	NA	NA
			Inter-laboratory CV (%)	
All laboratories	4.46	5.60	5.84	30.36

A: not possible to calculate a reliable mean LC50 due to the absence of lethality in the highest concentrations of the 3 runs; NA: CV could not be calculated; *LC50 from one run; CV: coefficient of variation

- At 48h and 96h, the intra-laboratory reproducibility of laboratories E and I could not be calculated, since there was insufficient lethality observed (except for one run in both laboratories at 96h) to derive a LC50. Both laboratories reported that they had problems dissolving prochloraz.
- For the two other laboratories (F and H), intra-laboratory reproducibility at 48h and 96h was acceptable (CVs <15%).
- The inter-laboratory reproducibility was acceptable at 48h (CV = 5.84%) and 96h (CV = 30.36%). It should be considered that the CV at 48h is based on the mean LC50 values of two laboratories.
- The mean LC50 values indicate that prochloraz is slightly less toxic at 96h.

1-Octanol

Analysis of 1-Octanol stock solutions and test concentrations

- P&G performed analytical measurements of the 1-octanol stock solutions and of the test concentrations for run 2 (for details see Annex IIIa).
- The average of the stock solution samples from the three independent runs was 90.0% of nominal concentration indicating the stock solution was accurately prepared.
- Geometric mean measured test concentrations throughout the test were 65.4-90.0% of nominal test concentrations. Substantial losses over the 24h renewal period were observed across all test concentrations.

LC50 values – 1-Octanol

The mean LC50 values of the three independent runs per laboratory are given in Table 13.

Table 13: 1-Octanol (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

1-Octanol	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory B	22.2	22.2	24.07	24.07
Laboratory F	19.2	19.2	4.93	4.93
Laboratory G	20.8	20.7	27.93	28.01
Laboratory H	20.8	20.6	11.81	13.31
			Inter-laboratory CV (%)	
All laboratories	20.75	20.68	5.87	5.88

CV: coefficient of variation

- The intra-laboratory reproducibility at 48h and 96h is acceptable (CVs <30%) and little variation between the two time points.
- The inter-laboratory reproducibility at 48h and 96h is acceptable (CVs <10%).
- Comparison of the mean LC50 values at 48h and 96h indicate no increase in toxicity.

Carbamazepine

Analysis of carbamazepine stock solutions and test concentrations

- Ipo-Pszczyna performed analytical measurements of the carbamazepine stock solutions and of the test concentrations for each run (for details see Annex IIIb).
- The measured stock solutions and test concentrations were >90% of the nominal value.

LC50 values – Carbamazepine

The mean LC50 values of the three independent runs per laboratory are given in Table 14.

Table 14: Carbamazepine (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Carbamazepine	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory D	161**	156	5.27	1.28
Laboratory E	177*	146	NA	6.13
Laboratory F	179	160	1.48	6.48
Laboratory K	189	152	15.86	3.39
			Inter-laboratory CV (%)	
All laboratories	177	153	6.37	3.80

NA: CV could not be calculated; *LC50 from one run; **mean LC50 from two runs; CV: coefficient of variation

- The intra-laboratory reproducibility at 48h (CVs <20%) is acceptable for three laboratories and at 96h (CVs <10%) for four laboratories.
- At 48h, the intra-laboratory reproducibility of laboratory E could not be calculated, since the LC50 could be derived for only one run.
- The inter-laboratory reproducibility is acceptable (CVs <10%) at 48h and 96h. It is nearly two-times lower (CVs <5%) at 96h.
- The mean LC50 values indicate that carbamazepine is slightly more toxic at 96h.

Triethylene glycol

The mean LC50 values of the three independent runs per laboratory are given in Table 15.

Table 15: Triethylene glycol (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Triethylene glycol	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory B	64500	52500	15.42	2.71
Laboratory E	76100	59700	6.52	9.65
Laboratory F	68100	52300	2.55	8.91
Laboratory I	76500	54500	7.91	1.59
			Inter-laboratory CV (%)	
All laboratories	71300	54800	8.38	6.26

CV: coefficient of variation

- The intra-laboratory reproducibility is acceptable for 48h (CVs <20%) and at 96h (CVs <10%). However, for two laboratories (F and E), the CV was higher at 96h than at 48h.
- The inter-laboratory reproducibility is acceptable at 48h and 96h (CVs <10%).
- Comparison of the mean LC50 values at 48h and 96h indicate an increase in toxicity by factor 1.3 at 96h.

Dimethyl sulfoxide

The mean LC50 values of the three independent runs per laboratory are given in Table 16.

Table 16: Dimethyl sulfoxide (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

Dimethyl sulfoxide	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory F	36800	36800	1.91	1.91
Laboratory H	48200	35000	12.05	3.67
Laboratory J	34600	31600	10.64	8.49
Laboratory K	41400	33100	7.77	7.75
			Inter-laboratory CV (%)	
All laboratories	40200	34100	14.88	6.58

CV: coefficient of variation

- The intra-laboratory reproducibility is acceptable at 48h (CVs <15%) and at 96h (CVs <10%).
- The inter-laboratory reproducibility is acceptable 48h (CV <15%) and at 96h (CV <10%). It is nearly two-times lower (CV = 6.58%) at 96h compared to 48h (CV = 14.88%).
- Comparison of the mean LC50 values at 48h and 96h indicate an increase in toxicity by factor 1.2 at 96h.

Overview of intra- and inter-laboratory reproducibility

Intra-laboratory reproducibility

Intra-laboratory reproducibility at 48h

A summary of the intra-laboratory reproducibility (CV%) calculated based on the mean LC50 values is given in Table 17.

Table 17: Intra-laboratory reproducibility - coefficients of variation for the LC50 values of 13 chemicals at 48h

		Laboratory (CV%)								
Time	**Chemical**	**B**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
48h	**Methylmercury (II) chloride**	-	5.1	-	0	-	-	-	3.7	-
	Copper (II) sulfate pentahydrate	-	-	-	14.9	12.9	9.9	16.5	-	-
	4,6-Dinitro-*o*-cresol	-	-	30.9*	27.8	-	9.7	-	-	26.5
	2,4-Dinitrophenol	-	20.6	-	1.2	-	-	23.6	-	8.4
	Merquat 100	-	-	-	NA	-	NA	-	NA	NA
	Luviquat HM 552	-	-	-	NA	-	NA	NA	-	NA
	Tetradecyl sulfate sodium salt	35.7*	-	-	4.5	32.8*	15.9	-	-	-
	Malathion	NA	-	-	14.4	-	-	-	NA	18.7
	Prochloraz	-	-	NA	11.8	-	8.9	NA	-	-
	1-Octanol	24.1	-	-	4.9	27.9	11.8	-	-	-
	Carbamazepine	-	5.3	NA	1.5	-	-	-	-	15.9
	Dimethyl sulfoxide	-	-	-	1.9	-	12.1	-	10.6	7.8
	Triethylene glycol	15.4	-	6.5	2.6	-	-	7.9	-	-

- : chemical not tested in the given laboratory (see also Table 3); CV: coefficient of variation; NA: CV could not be calculated; *: CV >30%

The intra-laboratory reproducibility at 48h:

- could not be calculated for two chemicals (Merquat 100, Luviquat HM 552) since LC50 values could only be derived in one run/chemical in the four laboratories;
- is not acceptable (CVs >30%) for two chemicals (4,6-dinitro-*o*-cresol and tetradecyl sulfate sodium salt) in two laboratories (3 CV values ranging from 30.9 to 35.7), whereas it is acceptable for these two chemicals in the other laboratories (5 CV values ranging from 4.5 to 27.8) and
- is acceptable (CVs <30%) for three chemicals (malathion, prochloraz and carbamazepine) in the laboratories where LC50 values could be derived for all runs (7 CV values ranging from 1.5 to 15.9%);
- is acceptable (CVs <30%) for the remaining six chemicals (23 CV values ranging from 1.2 to 27.9%).

Intra-laboratory reproducibility at 96h

A summary of the intra-laboratory reproducibility (CV%) calculated based on the mean LC50 values is given in Table 18.

Table 18: Intra-laboratory reproducibility - coefficients of variation for the LC50 values of 13 chemicals at 96h

		Laboratory (CV%)								
Time	**Chemical**	**B**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
96h	**Methylmercury (II) chloride**	-	11.2	-	3.5	-	-	-	2.1	-
	Copper (II) sulfate pentahydrate	-	-	-	14.9	12.9	9.2	7.2	-	-
	4,6-Dinitro-*o*-cresol	-	-	1.7	14.3	-	12.4	-	-	18.5
	2,4-Dinitrophenol	-	32.7*	-	16.8	-	-	1.6	-	5.3
	Merquat 100	-	-	-	17.1	-	26.7	-	37.2*	1.9
	Luviquat HM 552	-	-	-	7.8	-	11.6	13.5	-	9.1
	Tetradecyl sulfate sodium salt	34*	-	-	4.5	28.2	15.9	-	-	-
	Malathion	19	-	-	8.8	-	-	-	10.3	33.2*
	Prochloraz	-	-	NA	11.3	-	8.7	NA	-	-
	1-Octanol	24.1	-	-	4.9	28	13.3	-	-	-
	Carbamazepine	-	1.3	6.1	6.5	-	-	-	-	3.4
	Dimethyl sulfoxide	-	-	-	1.9	-	3.7	-	8.5	7.8
	Triethylene glycol	2.7	-	9.7	8.9	-	-	1.6	-	-

- : chemical not tested in the given laboratory (see also Table 3); CV: coefficient of variation; NA: CV could not be calculated; *: CV >30%

The intra-laboratory reproducibility at 96h:

- could not be calculated for one chemical (prochloraz) in two laboratories since LC50 values could only be derived in one run/chemical. In laboratory E (where the analytics are performed) there was strong evidence that prochloraz was not well dissolved in the stock solution (<70% of nominal concentration for one run). Laboratory I reported difficulties in dissolving prochloraz for each run.
- is not acceptable (>30%) for four chemicals (2,4-dinitrophenol, Merquat 100, tetradecyl sulphate sodium salt, malathion) in four laboratories (4 CV values ranging from 32.7 to 37.2%)
- is acceptable (CV <30%) for the remaining nine chemicals (45 CV values ranging from 1.3 to 28.2%).

Inter-laboratory reproducibility

A summary of the inter-laboratory reproducibility (CV%) calculated based on the mean LC50 is given in Table 19.

Table 19: Inter-laboratory reproducibility - coefficients of variation (CV) for the LC50 values of 13 chemicals

Chemicals	**CV (%)**		**N**
	48h	**96h**	
Methylmercury (II) chloride	46.9*	50.2*	3
Copper (II) sulfate pentahydrate	41.7*	33.6*	4
4,6-Dinitro-*o*-cresol	2.8	7.5	4
2,4-Dinitrophenol	23.5	22.7	4
Merquat 100	NA	40.8*	4
Luviquat HM 552	NA	24.8	4
Tetradecyl sulfate sodium salt	25	25.8	4
Malathion	25.8	13	4
Prochloraz	5.8	30.4*	4
1-Octanol	5.9	5.9	4
Carbamazepine	6.4	3.8	4
Dimethyl sulfoxide	14.9	6.6	4
Triethylene glycol	8.4	6.3	4

N: number of laboratories that tested the chemical; CV: coefficient of variation;
NA: CV could not be calculated; *: CV >30%

The inter-laboratory reproducibility at 48h:

- is acceptable for nine chemicals with CVs ranging from 2.8 to 25.8%.
- not acceptable (CVs >30%) for two chemicals (methylmercury (II) chloride, copper (II) pentahydrate sulfate). A factor contributing to the large CVs is the very high acute toxicity of the chemicals. Small differences in the LC50 values are magnified resulting in larger CVs.
- could not be calculated for two chemicals (Merquat 100, Luviquat HM 552). As previously said, insufficient lethality was observed at 48h for these polymers since they did not pass the chorion due to their physical-chemical properties.

The inter-laboratory reproducibility at 96h:

- is acceptable for nine chemicals with CVs ranging from 3.8 to 25.8%.
- is not acceptable (CVs >30%) for three chemicals (methylmercury (II) chloride, copper (II) pentahydrate sulphate, Merquat 100). Similar statistical considerations as for 48h apply here.
- is not acceptable (CV >30%) for one chemical (prochloraz). It should be noted that in two laboratories the highest test concentration did not trigger 50% lethality.

EVALUATION OF TIME-DEPENDENT CHANGES IN LC50S

ZFET LC50 values were calculated for 24, 48, 72 and 96h to assess the time-dependent changes in toxicity for Phase 2 chemicals. Based on these determinations it may be possible to develop recommendations to perform the ZFET at durations shorter than 96h for certain groups of chemicals.

The evaluation reveals the following findings:

- Four relatively distinct temporal patterns of toxicity were identified in the chemicals tested. These were: (Group 1) chemicals whose toxicity was observed primarily early in exposure, (Group 2) chemicals whose toxicity continues to steadily progress throughout the exposure, (Group 3) chemicals whose toxicity rapidly changes after 24h, and (Group 4) chemicals whose toxicity is mostly expressed following hatch at 72h. These remain relatively arbitrary groupings and indicate trends, and should not be over-interpreted.
- There is no clear pattern of chemical category, functional use, mode of action or potency that is associated with any grouping of chemicals using temporal patterns of LC50s as a guide.
- Some chemicals possess properties that would result in erroneous assessments of overall potential to be toxic to fish if the ZFET would be terminated before hatch. Only one chemical category, the cationic polymers, may be typified as consistent members of this group. Other members may eventually be indicated by physical-chemical properties such as possessing low solubility and high hydrophobicity (high log Kow), or whose potency is very close to the limits of solubility.

A detailed report on this evaluation is included as Annex VIII.

EVALUATION OF THE HATCHING RATE

In addition to the four lethal effects, the laboratories had been asked to record the hatching. This information was used to calculate the overall hatching rate in the negative control and compares whether inter-laboratory differences for hatching may have affected LC50 determinations.

The evaluation reveals the following findings:

- Negative control hatch rates for zebrafish embryos are high and quite consistent. Over 80% of the negative control zebrafish hatch by 72h and the 90th percentile exceeds 90% hatch by 96h.
- 80% hatch at 96h was not achieved in three out of 153 runs (each in different laboratories).
- The 96 h LC50 appears to be unrelated to the percent of embryos hatched at 72h.

A detailed report on this evaluation is included as Annex IX.

IMPACT OF THE GROUP SIZE ON THE ESTIMATION OF LC50 IN THE ZFET

For the purpose of the study, 20 zebrafish embryos per group were used in a series of five concentrations. A statistical computer simulation study was performed to quantify the effect of group test size on the estimation of the LC50 and its confidence interval (for details see Annex X). The simulations demonstrate that the likelihood of deriving a sound LC50 with reasonably useful 95% confidence intervals is diminished for smaller group sizes, in particular when the response trend is relatively flat and when the true (but unknown) LC50 is not well centered in the concentration range chosen.

It is therefore recommended that the group size of 20 embryos should be maintained for the ZFET to ensure the value of the test. In practical terms, this also has the benefit of reducing the likelihood that if fewer embryos/group are used multiple (non-random) exposure concentrations would be employed on the same exposure plate (assuming a 24-well plate is used).

COMPARISON OF ZFET AND FISH LC50 VALUES

For the comparison of ZFET LC50 values and fish LC50 values, 96h acute fish toxicity data were retrieved from the literature and the OECD QSAR toolbox (Version 2.0). Table 20 is meant to give a preliminary idea of the predictive capacity of the ZFET test for acute fish toxicity.

Table 20:Comparison of ZFET LC50 values and the 96h acute fish LC50 values

Phase 2b chemicals	**ZFET mean LC50 (mg/L)**		**Fish acute* LC50 (mg/L)** min - **mean** - max
	48h	**96h**	**96h**
Methylmercury (II) chloride	0.042	0.028	0.031 - **0.145** - 0.46 (6)**
Copper (II) sulfate pentahydrate	0.308	0.291	0.008 - **0.224** - 0.749 (11)
4,6-Dinitro-*o*-cresol	0.723	0.567	0.066 - **0.863** - 2.2 (7)
2,4-Dinitrophenol	4.123	3	0.39 - **6.843** - 27.1 (19)
Merquat 100	NA	0.496	6.52 (1)
Luviquat HM 552	NA	0.876	0.748 (1)
Tetradecyl sulfate sodium salt	0.337	0.339	2.5 - **3.031** - 3.55 (3)
Malathion	6.123	4.56	0.003 - **0.289** - 25 (47)
Prochloraz	4.461	5.6	0.53 - **0.583** - 0.68 (3)
1-Octanol	20.7	20.675	13 - **15.68** - 24 (10)
Carbamazepine	177	153	43 (1)
Triethylene glycol	71300	54800	34000 - **40429** - 52000 (6)
Dimethyl sulfoxide	40200	34100	59900 - **71251** - 92500 (5)

*Measured fish LC50 values were retrieved from literature and the OECD QSAR toolbox (Version 2.0).

**: Numbers in brackets represent the number of studies. NA: LC50 could not be calculated

Using the fish toxicity categories (non-toxic >100mg/l; moderately toxic = 10 -100 mg/l; toxic = 1 -10 mg/l; very toxic <1 mg/l), the comparison of fish LC50 and ZFET LC50 96h reveals that:

- the two chemicals (triethylene glycol, dimethyl sulfoxide) non-toxic to fish are also non-toxic in the ZFET;
- four chemicals (methylmercury (II) chloride, copper (II) sulfate pentahydrate, 4,6-dinitro-*o*-cresol, Luviquat HM 552) very toxic to fish are also very toxic in the ZFET;
- the toxicity of two chemicals (2,4-dinitrophenol, 1-octanol) is in the same range for ZFET and fish;
- the three chemicals with specific mode of action (malathion, prochloraz and carbamazepine) (see Annex II) are less toxic to zebrafish embryos than to juvenile fish. In addition, it should be noted that prochloraz and carbamazepine have very limited fish data.
- the toxicity of two chemicals (Merquat 100, tetradecyl sulfate sodium salt) is higher in the ZFET compared to fish.

When using ZFET LC50 48h data, the comparison does not reveal differences for 11 out of 13 chemicals. However, the comparison cannot be done for Merquat 100 and Luviquat HM 552, since LC50 could not be calculated at 48h.

CONCLUSIONS PHASE 2

- Regarding transferability, intra- and inter-laboratory reproducibility, the VMG concludes that:
  - the ZFET was successfully transferred to four additional laboratories; and
  - the intra- and inter-laboratory reproducibility of the ZFET is in general acceptable (CV <30%) regardless of the chemical or the laboratory. Nevertheless, it is noted that for three chemicals (Merquat 100, methylmercury (II) chloride, copper (II) sulfate pentahydrate) CVs >30% were calculated. However, a factor contributing to the large CVs is the very high acute toxicity of these chemicals, since relatively small differences in the LC50 values are magnified and result in a larger CV. With prochloraz tested close to its limit of solubility acceptable intra- and inter-laboratory reproducibility was achieved only in two laboratories.

- Regarding the analytical measurements performed in two laboratories, the VMG concludes that:
  - the determination of exposure concentrations in the ZFET can be accomplished by modern analytical methods, even when very low sample volumes and highly toxic substances are involved, and
  - the most challenging chemicals were characterized by combinations of low solubility, high biodegradability, and being semi-volatile. Results for challenging chemicals appear to be mostly related to the chemistry of the chemical and not a function of the exposure system.

- Regarding the 48h vs 96h exposure, the VMG concludes that:
  - as expected the chorion acts as a barrier for chemicals with high molecular weight, i.e. for the two polymers tested with the ZFET (Merquat 100 and Luviquat HM 552) toxicity was only observed post-hatch when the assay duration is extended to 96 hours of exposure; and
  - the other chemicals tested were slightly more toxic at 96h than at 48h, however, LC50 remained in the same order of magnitude.
  - Based on these observations a 96-hour exposure is recommended.

- Regarding the evaluation of time-dependent changes in LC50s, the VMG concludes that:
  - for the 20 chemicals tested in Phase 1 and Phase 2, there is no clear pattern of chemical category, functional use, mode of action or toxicity that is associated with any grouping of chemicals using temporal patterns of LC50s as a guide; and
  - the toxicity of some chemicals would have been underestimated, if the tests would have been terminated before hatch, e.g. for cationic polymers.

- Regarding the hatching rate, the VMG concludes that:
  - the hatching rate in the negative control in Phase 2b was consistent and it might be useful to include as additional acceptance criteria for the ZFET that the hatching rate in the negative control should exceed 80% at 96h.

- Regarding the number of embryos/test concentration the VMG concludes that:
  - the group size of 20 embryos per test concentration should be maintained to ensure sound estimation of the LC50 values.

- Regarding predictive capacity, the VMG concludes that:

- For the 13 chemicals tested in Phase 2b, the predictive capacity of ZFET for acute fish toxicity is very promising but will need to be underpinned with additional data, e.g. based on the results of the data compilation of fish embryo toxicity data versus fish acute data performed by S. Belanger, J. Rawlings, G. Carr (*"An Update to the Fish Embryo Toxicity-Acute Fish Toxicity Relationship and Prospects for Support of the Use of the FET as an Animal Alternative"*) and provided as a separate document to the OECD.

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LIST OF ANNEXES