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IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
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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 I** of the validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test (ZFET), on transferability, intra-, and inter-laboratory reproducibility for seven chemicals. It includes five annexes. Four additional annexes are included in **Part II** of the report. The nine annexes of the report are as follows:

- Annex I: Study Documents and Method Description
- Annex II: Analysis of 3,4-DCA Concentrations in Fish Embryo Test Stock and Exposure Solutions
- Annex III: Statistical Report Phase 1a: Single Run with 3,4-DCA
- Annex IV: Statistical Report Phase 1a: Three Runs with 3,4-DCA
- Annex V: Analysis of 6 chemicals in Fish Embryo Test Stock and Exposure Solutions for Phase 1b
- Annex VI: Statistical Report Phase 1b- Six chemicals
- Annex VII: Trial Plan for Phase 1a - Transferability
- Annex VIII: Trial Plan for Phase 1b – Testing of six chemicals
- Annex IX: Standard Operating Procedure

The Zebrafish Embryo Toxicity Test (ZFET) was developed by the German Federal Environment Agency (UBA). The validation report (Phase 1) was prepared by the European Commission (EC-ECVAM), and endorsed by the Working Group of National Coordinators of the Test Guidelines Programme at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology (Joint Meeting) agreed to its declassification on 5 August 2011.

This document is published under the responsibility of the Joint Meeting.

Report of the Test Method Validation for the Zebrafish Embryo Toxicity Test (ZFET)

Phase 1 –
Transferability, intra- and inter-laboratory reproducibility for 7 chemicals

16th March 2011

As agreed by the Validation Management Group

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SUMMARY

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

In this first phase of the study, the aim was to evaluate the transferability, and the intra- and inter-laboratory reproducibility of the Zebrafish FET (ZFET) with seven chemicals that spanned a wide range of toxicity and various modes of action. The chemicals were tested at five different concentrations in three independent runs in at least four laboratories with appropriate controls. Stock solutions and test concentrations of at least one laboratory are analytically confirmed.

Newly fertilised zebrafish eggs were exposed for up to 96h to chemicals. 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.

For this first phase, the VMG concluded that the ZFET test was successfully transferred from the lead laboratory to the participating laboratories.

The intra- and inter-laboratory reproducibility of the LC50 values is promising. In general, intra-laboratory variability is low with the vast majority of coefficients of variation (CV) for all chemicals below 30%. Inter-laboratory variability is higher and ranged from 4.78 to 58% at 96h although for 5 of the 7 chemicals the values were between 4.78 and 23.6%. The greatest CVs were for difficult test chemicals. Analytical measurements performed in one laboratory confirmed nominal concentrations of the stock solutions and test concentrations except for 2 chemicals which possess properties associated with difficult test substances.

INTRODUCTION

1. In autumn 2005, the German Federal Environment Agency (UBA) submitted the draft guideline "Fish embryo toxicity (FET) test" to the OECD Test Guideline Programme (Project 2.7) together with a Draft Detailed Review 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 ZFET, however, data providing sufficient evidence for the reproducibility of the method were lacking.

2. 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 will be tested.

3. The Phase 1a study was conducted in two steps from April to October 2009. 3,4-Dichloroaniline (3,4-DCA) was used as test chemical since it is well established as a positive control in the Fish Egg Toxicity test for waste water testing (DIN 2001). In the first step, the seven participating laboratories evaluated the transferability of the Standard Operation Procedure (SOP) by testing 3,4-DCA in six test concentrations. In the second step, the laboratories carried out three independent runs with the same test concentrations. The results were used to determine the concentration of the positive control (3,4-DCA) for further tests (for detailed study design see Section 7.).

4. The Phase 1b study was conducted from November 2009 to October 2010. Six laboratories trained in Phase 1a tested six chemicals in three independent runs following a slightly revised SOP. 3,4-DCA was used as positive control at a concentration of 4.0 mg/L (for detailed study design see Section 8.).

Validation Management Group

5. 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	JRC/IHCP/ECVAM Ispra, ITALY	Coordination/reporting
André Kleensang	JRC/IHCP/ECVAM Ispra, ITALY	Data analysis for Phase 1a
Patric Amcoff	OECD Environment, Health and Safety Division, Environment Directorate Paris, FRANCE	OECD Test Guideline Programme
Thomas Braunbeck	University of Heidelberg Heidelberg, GERMANY	Lead laboratory & Umweltbundesamt 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
Adam Lillicrap	NIVA Oslo, NORWAY	Independent adviser
Susanne Walter-Rohde	Umweltbundesamt, Dessau-Roßlau, GERMANY	Lead country OECD project 2.7 (joined the VMG in April 2010)

Participating Laboratories

Laboratory	Responsible
University of Heidelberg, Heidelberg, GERMANY[1]	Prof. Dr. Thomas Braunbeck
Procter & Gamble, Cincinnati, OH, USA[2]	Scott Belanger, PhD
Ipo-Pszczyna, Pszczyna, POLAND[3]	Przemysław Fochtman, PhD
IVM, Amsterdam, THE NETHERLANDS	Juliette Legler, PhD
UFZ, Leipzig, GERMANY	Stefan Scholz, PhD
RIVM, Bilthoven, THE NETHERLANDS	Leo van der Ven, PhD
VITO, Mol, BELGIUM	Hilda Witters, PhD

[1] Lead laboratory

[2] Chemical analysis as described in Sections 7 and 8.

[3] Ipo-Pszczyna could only participate in Phase 1a

Definition of the SOP

6. Before the start of the study the lead laboratory provided a draft SOP, which was reviewed by the VMG and discussed with the participating laboratories before the start of Phase 1a.

7. The SOP deviates from the latest version of the OECD draft guideline (status May 2008) in relation to the following points, taking into consideration the concerns expressed by the *ad hoc* Expert Group:

- Possibility that the chorion could act as a barrier to chemical exposure, therefore the exposure duration was extended beyond hatch (96h) with calculation of LC50 at 48h and 96h
- Number of embryos per concentration and control were increased to 20 embryos instead of 10 embryos
- An acceptance criterion was set for the fertilisation rate.

Chemicals and test concentrations

8. Chemicals were selected based on the recommendations of the *ad hoc* Expert Group (see Minutes of the meeting in May 2008). The University of Heidelberg purchased and distributed 3,4-DCA to the laboratories, whereas ECVAM purchased, aliquoted and distributed the six chemicals tested in Phase 1b.

9. The lead laboratory (University of Heidelberg) and one participating laboratory (Procter & Gamble, P&G) performed the range-finding tests for Phase 1b. Since it was not possible to determine an LC50 value for 2,2,6,6-Tetramethyl-4-piperidone (20% lethality with 0.9 g/L after 96h), the VMG decided to test 6-Methyl-5-heptene-2-one, a chemical with similar properties and toxicity to fish.

10. Table 1 lists the test chemicals and concentrations. More detailed information on the chemicals is given in the respective trial plans (not included in this report but available on request).

Table 1: Physical chemical properties and test concentrations of the ZFET chemicals for Phase 1

Chemical	**Fish Toxicity**	**CAS Number**	**Catalogue Number**	**Lot Number**	**MW (g/mol)**	**Log Kow**	**HLC (Pas-m3/mole)**	**Solubility (mg/L)**	**Test Concentrations**
Triclosan	+++	3380-34-5	72779	1412854	289.55	4.76	0.00051	4.621	0.075, 0.15, 0.3, 0.6, 1.2 mg/L
Dibutyl maleate	++	105-76-0	D47102	07715ch	228.29	4.16	0.0768	8.709	0.25, 0.5, 1, 2, 4 mg/L
2,3,6-Trimethylphenol	++	2416-94-6	92693	1290095	136.2	3.15	0.399	1580	8, 12, 18, 27, 40.5 mg/L
3,4-	++	95-76-1	35827	6080x	162.02	2.69	0.19	337.9	0.5, 1, 2, 3.7, 4, 8

Dichloroaniline									mg/L
6-Methyl-5-heptene-2-one	+	110-93-8	67320	S52972-429	126.2	2.06	21.5	4364.1	25, 42.5, 72.25, 122.825, 208.03 mg/L
Sodium chloride	-	7647-14-5	S7653	106K0081	58.44	-0.46	3.580E-025	359000	1, 2, 4, 8, 16 g/L
Ethanol	-	64-17-5	34923	sze91380	46.07	-0.31	0.574	1×10^6	5.3, 8, 12, 18, 27 g/L

- = non-toxic (LC50>100 mg/L); + = moderately toxic (LC50 from 10 to 100 mg/L); ++ = toxic (LC50 from 1 to 10 mg/L; +++ = very toxic (LC50<1 mg/L); MW = Molecular Weight; HLC = Henry's Law Constant. All chemicals were purchased from Sigma-Aldrich; in Phase 1b, Laboratory C used sodium chloride from a different supplier to perform run nº3. Note log K_{ow}, HLC, and solubility were estimated using EPISUITE 4.0 (2008) except when measured values were available (cited within EPISUITE).

Phase 1a – Transfer of the SOP

Study design

11. In a first step, the seven participating laboratories evaluated the transferability of the SOP by testing 3,4-DCA in six concentrations (0.5, 1, 2, 3.7, 4, and 8 mg/L plus negative control). For further details see Annex I.

12. As described below, the results of this first experiment led to an amendment of the SOP and the trial plan. In the second step, three independent runs were performed with the six concentrations. "Independent run" means that the experiments were performed with different batches of zebrafish eggs, on different days and with newly prepared test concentrations.

13. 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.

14. P&G performed analytical measurements of the stock solutions of the participating laboratories and their own 3,4-DCA test concentrations.

15. Since the analytical measurements demonstrated a substantial loss (>20%) of the 3,4-DCA concentration in the first experiment, the SOP was modified as follows: a) test vessels and 24-well plates were pre-saturated with the respective test concentrations at least 24h before the start of the test, and b) daily renewal of the test concentrations and controls was required in order to maintain the test concentration >80% during the exposure period, which corresponds to the semi-static method as defined in OECD TG 203 (OECD, 1992).

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

17. With regard to intra- and inter-laboratory reproducibility, the VMG agreed upon that coefficients of variation (CV) below 30% would be acceptable.

Results

18. The laboratories provided the data to the coordinator using the corresponding reporting templates (see Annex I). Prior to statistical analysis, 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 SOPs (The summary of the quality check is available on request).

Analysis of 3,4-DCA stock solutions and test concentrations

19. The detailed report of the analytical measurements is attached as Annex II.

20. Due to problems with the shipment of the 3,4-DCA stock solutions to the P&G laboratory (USA), stock solutions of only four laboratories could be analysed. No substantive differences between laboratories for stock solutions were detected.

21. The analytical measurement of the test concentrations used in the P&G laboratory demonstrated for the single run with 3,4-DCA a substantial loss (>20%) of 3,4-DCA concentration at the end of the test. The test concentrations of the three runs with 3,4 DCA were remarkably similar to that of the single run, despite the daily renewal introduced after the observed loss. CVs ranged from 0.9-9.9% across all exposure concentrations. An explanation for this could be the overestimated stability (up to 6 months) of the stock solution. As the results on the stability testing (see Annex II, Table 1) show, a decrease in the concentration of the stock solution was already evident after 3 months.

LC50 values - Single run with 3,4 DCA

22. The seven runs met the acceptance criteria. Table 2 gives an overview on the LC50 values and confidence intervals calculated on the basis of the data provided by the seven laboratories. Two statistical models were used to evaluate the confidence interval at 48h and 96h (The detailed report of the statistical analysis is attached as Annex III).

Table 2: Single run with 3,4-DCA - LC50 values and confidence intervals of the Zebrafish Embryo Toxicity Test

48h	Log-logistic with LC50 as parameter				Log-logistic with log(LC50) as parameter			
Lab	LC50 [mg/L]	95%CI -	95%CI +	Model fit	LC50 [mg/L]	95%CI -	95%CI +	Model fit
A	5.3	4.4	6.2	not reliable *	5.3	4.5	6.3	not reliable*
B	1.8	1.5	2.0	ok	1.8	1.5	2.0	ok
C	1.5	1.3	1.8	ok	1.5	1.3	1.8	ok
D	2.3	1.9	2.6	not reliable **	2.3	1.9	2.7	not reliable **
E	3.1	2.4	3.7	ok	3.1	2.5	3.8	ok
F	2.7	2.2	3.3	ok	2.7	2.3	3.3	ok
G	3.5	3.2	3.9	ok	3.5	3.2	3.9	ok
All	2.7	2.5	2.8	ok	2.7	2.5	2.8	ok

96h	Log-logistic with LC50 as parameter				Log-logistic with log(LC50) as parameter			
Lab	LC50 [mg/L]	95%CI -	95%CI +	Model fit	LC50 [mg/L]	95%CI -	95%CI +	Model fit
A	4.4	3.6	5.1	not reliable *	4.4	3.7	5.2	not reliable *
B	1.8	1.6	2.1	ok	1.8	1.6	2.1	ok
C	1.5	1.2	1.7	ok	1.5	1.3	1.7	ok
D	2.3	1.9	2.6	not reliable **	2.3	1.9	2.7	not reliable **
E	3.0	2.3	3.7	ok	3.0	2.4	3.8	ok
F	2.5	2.1	3.0	ok	2.5	2.1	3.0	ok
G	3.4	3.0	3.8	ok	3.4	3.0	3.8	ok
All	2.5	2.3	2.7	ok	2.5	2.3	2.7	ok

CI: confidence interval; *: toxicity only evident at highest concentration: **: bad curve fitting; Note: Detailed statistical report is given in Annex III

- The LC50 values were consistent (within a factor of 2) in six out of seven laboratories at 48h and 96h.
- Laboratory A reported a deviation from the SOP; i.e. the test concentrations had not been freshly prepared but 24h before starting the test. This might explain why lethality was only observed at the highest concentration and in consequence the lower lethality observed at 48h (LC50 5.3 mg/L) and 96h (LC50 4.4 mg/L).
- It should be noted that the 48h LC50 values for 3,4-DCA were lower than the LC50 value of 3.7 mg/L given in the German DIN guideline for waste water testing (DIN 2001).
- A slightly higher toxicity of 3,4-DCA is observed after 96h exposure.

LC50 values - Three runs with 3,4 DCA

Note: As described in 7.1, modifications to the SOP became necessary due to the substantial loss of 3,4-DCA over the course of the test. These modifications are:

- test vessels and 24-well plates need to be pre-saturated with the respective test concentrations at least 24h before the start of the test, and
- use of a semi-static method, i.e. daily renewal of the test concentrations and controls is mandatory in order to maintain the test concentrations >80% of the nominal concentration during the exposure period.

23. These modifications are consistent with provisions for less stable substances in the existing OECD TG 203 (OECD, 1992).

24. Five out of seven laboratories provided a complete data set i.e. their three runs with 3,4-DCA met the acceptance criteria as defined in the SOP. From the data sets submitted by the other two laboratories, two runs did not meet the acceptance criteria since:

- the incubation temperature for run n°2 of laboratory A was not within the defined range; and
- the overall survival rate for the negative control was ≤90% for run n°2 of laboratory B.

25. The LC50 values of the three independent runs per laboratory are given in Table 3 (the detailed report of the statistical analysis is available in Annex IV).

Table 3: Three runs with 3,4-DCA: LC50 values and confidence intervals of the Zebrafish Embryo Toxicity Test

		LC50 3,4-Dichloroaniline [mg/L]			
Laboratory	**Run**	**48h**	**95%CI+-**	**96h**	**95%CI+-**
A	1	2.2	1.2	2.1	1.0
	2	not qualified		not qualified	
	3	5.4	0.8	5.1	0.7
combined A	1,3	3.7	0.3	3.5	0.4
B	1	1.0	0.2	1.1	0.2
	2	not qualified		not qualified	
	3	1.5	0.3	1.4	0.2
combined B	1,3	1.2	0.2	1.2	0.1
C	1	3.7	0.2	2.5	0.4
	2*	3.8	0.3	3.3	0.5
	3	3.3	0.4	2.5	0.3
combined C	1,2,3	3.1	0.3	2.4	0.2
D	1	3.1	0.4	2.6	0.3
	2	2.5	0.4	2.4	0.3
	3*	2.7	11.1	2.7	10.6
combined D	1,2,3	2.8	0.2	2.6	0.2
E	1	5.4	0.8	4.8	0.7
	2	4.5	0.7	4.1	0.6
	3	3.6	0.7	3.3	0.6
combined E	1,2,3	4.5	0.4	4.1	0.4
F	1	2.5	0.4	2.3	0.3
	2	2.8	0.5	2.4	0.4
	3	3.9	0.5	3.2	0.4
combined F	1,2,3	3.0	0.3	2.6	0.2
G	1	3.3	0.4	2.8	0.4
	2	4.5	1.0	3.9	0.2
	3	4.1	0.3	3.6	0.3
combined G	1,2,3	4.3	0.3	3.4	0.2
Overall	19 runs	3.2	0.1	2.7	0.1

CI: confidence interval
****: further details are given in Annex IV Table 1***

- The combined LC50 values from the laboratories ranged from 1.2 to 4.5 mg/L at 48h and from 1.2 to 4.1 mg/L at 96h. As reported for the single run, the toxicity of 3,4-DCA increased with increasing exposure time in all laboratories.

Table 4 shows the intra- and inter-laboratory reproducibility of the LC50 values.

Table 4: Three runs with 3,4-DCA: Combined LC50 values and intra-laboratory and inter-laboratory reproducibility of the ZFET

3,4-DCA	**Combined LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory A	3.7*	3.5*	58.8*	58.5*
Laboratory B	1.2*	1.2*	27.2*	17.1*
Laboratory C	3.1	2.4	7.3	16.6
Laboratory D	2.8	2.6	10.0	4.4
Laboratory E	4.5	4.1	20.4	18.9
Laboratory F	3.0	2.6	24.6	17.9
Laboratory G	4.3	3.4	14.9	17.2
			Inter-laboratory CV (%)	
All laboratories	3.2	2.7	*33.7*	*33.4*
Five laboratories (C-G) with 3 runs	n.c.	n.c.	*22.1*	*23.6*

*** = based on two runs¸ n.c. = not calculated**

- The intra-laboratory reproducibility of the five laboratories with three qualified runs is acceptable at 48h and 96h (CV<30%). The CVs of laboratory A and B are only indicative since they are calculated for two runs, nevertheless, it should be noted that the reproducibility in Laboratory A is not acceptable.
- Considering only the results of the laboratories with three qualified runs, the inter-laboratory reproducibility is acceptable (CV < 30%)
- The ratio of the highest to lowest LC50 for laboratories with three qualified runs was 1.6 and 1.7, at 48 and 96 hrs respectively.

CONCLUSIONS PHASE 1A

26. Despite the fact that two laboratories provided only two qualified runs, the VMG concluded that the ZFET could be successfully transferred from the lead laboratory to the six participating laboratories. The problems associated with the two non-qualified runs were addressed and could be clarified during discussions with the respective laboratories.

27. The data of the three independent runs with 3,4-DCA indicate a promising intra- and inter-laboratory reproducibility; however, more data are needed to draw sound conclusions.

28. As indicated by the statistician during the planning of the study, it was necessary to establish a concentration for the positive control, which would cause a higher mortality than the one given in the OECD draft FET guideline (10%). Based on the 3,4-DCA LC50 values, the statistician concluded that a concentration of 4.0 mg/L would result in 80% lethality over 96h exposure and therefore, could serve as positive control in future experiments. In consequence, the VMG set the acceptance criteria for the positive control, i.e. the test is acceptable if the positive control (4.0 mg/l 3,4-DCA) shows at least 30% mortality after 96h exposure.

29. The analytical results showed that the 3,4-DCA stock solution should only be stored up to 2 months, since the results on the stability testing of the 3,4-DCA stock solution revealed a decrease in the concentration after 3 months.

30. The VMG decided to continue with the pre-saturation of the test vessels and 24-well plates with the respective test concentrations at least 24h before the start of the test and daily renewal of the test concentrations and controls in order to maintain the test concentrations >80% during the exposure period.

31. The feedback of the laboratories and the results of the analytical measurements were used to improve the SOP for Phase 1b (see (8.1).

PHASE 1B – TESTING OF SIX CHEMICALS

Study design

32. As described for Phase 1a, the six 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, temperature and conductivity were performed for the controls and the highest concentration as described in the respective SOP.

33. The results of Phase 1a led to two amendments to the SOP:

- A minimum microscopic magnification of 80x should be used for the detection of the heart beat.
- The acceptance criteria for the positive control (3.4-DCA) was included: 4.0 mg/L 3,4-DCA should result in a minimum mortality of 30 %.

34. P&G carried out the analytical measurement of the six chemicals tested in their laboratory by measuring the stock solutions and the test concentrations of one run per chemical.

35. The laboratories were asked to store samples of the stock solutions of the four fish toxic chemicals, since it might be necessary to confirm their concentration.

36. Each laboratory measured the concentration of the sodium chloride stock solution. Ethanol was directly used to prepare the test concentrations and there was no need to prepare stock solutions.

37. LC50 values were calculated for 48h and 96h exposure times 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 VI.

38. 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.

39. Since not all laboratories had the capacity to test all chemicals, the VMG decided to distribute the six chemicals amongst the laboratories as given in Table 5. This distribution ensured that each chemical was at least tested in four laboratories.

Table 5: Distribution of chemicals over the six laboratories

		Laboratories*					
Toxicity	**Chemicals**	**A**	**B**	**C**	**D**	**F**	**G**
Very toxic to fish *(LC50 < 1 mg/L)*	Triclosan		X	X		X	X
Toxic to fish (*LC50 from 1 to 10 mg/L)*	Dibutyl maleate	X		X	X	X	X
Moderately toxic to fish (*LC50 from 10 to 100 mg/L)*	2,3,6-Trimethylphenol	X		X	X	X	X
	6-Methyl-5-heptene-2-one		X	X		X	X
Non-toxic to fish *(LC50 > 100 mg/L)*	Sodium chloride		X	X		X	X
	Ethanol	X		X	X	X	X

*: Laboratory E did not participate in Phase 1b

Results

40. The laboratories provided data of 81 runs to the coordinator using the updated reporting template (see Annex I). Prior to statistical analysis, the data underwent a quality check by the coordinator and an independent person.

41. Out of the 81 runs, four runs did not qualify, i.e. three runs of Laboratory C did not meet the acceptance criteria since the lethality in the negative external control was >10% and Laboratory D reported a mistake in the preparation of the test concentration for one run. In consequence:

- Laboratory C repeated the failed runs (two with sodium chloride and one with ethanol).
- Laboratory D repeated one run with dibutyl maleate.

42. The repeated runs met the acceptance criteria.

In the following, 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 Annex VI.

Triclosan

Analysis of Triclosan stock solutions and test concentrations

43. P&G performed analytical measurements of the Triclosan stock solutions and of the test concentrations for one run n° 1 (for details see Annex V).

- There was no difference between the nominal and the measured stock solution concentrations.
- The measured test concentrations were between 90 to 100% of the nominal test concentrations until the end of the test.

LC50 values – Triclosan

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

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

Triclosan	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory B	0.418	0.355	2.14	16.79
Laboratory C	0.384	0.283	14.15	37.99
Laboratory F	0.396	0.275	8.68	5.86
Laboratory G	0.471	0.302	17.73	2.6
			Inter-laboratory CV (%)	
All laboratories	0.417	0.304	9.24	11.8

- The intra- and inter-laboratory reproducibility at 48h and 96h is acceptable in all laboratories with the exception of laboratory C at 96h.
- The ratio of the highest mean LC50 value to the lowest is 1.22 and 1.29 for 48h and 96h, respectively.
- Comparison of the mean LC50 values at 48h and 96h indicate an increase in toxicity by factor 1.5.

Dibutyl maleate

Analysis of Dibutyl maleate stock solutions and test concentrations

P&G performed analytical measurements of the Dibutyl maleate stock solutions and of the test concentrations for run n° 2 (for details see Annex V).

- There was no difference between the nominal and the measured stock solution concentrations.
- The measured test concentrations were significantly lower (between 30 to 40%) than the nominal test concentrations during the exposure. It should be noted that this chemical has a log K_{ow} >4 and is therefore only moderately soluble (~173 mg/L) and fairly sorptive. Importantly, it is also ready biodegradable with >90% Dissolved Organic Carbon (DOC) loss at 14 days in ready tests (OECD, 2005). Losses are potentially accounted for due to a combination of degradation, sorption, and possibly even metabolism since the compound is a simple di-ester.

LC50 values – Dibutyl maleate

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

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

Dibutyl Maleate	Mean LC50 (mg/L)		Intra-laboratory CV (%)	
	48h	**96h**	**48h**	**96h**
Laboratory A	1.250	0.807	10.27	19.16
Laboratory C	1.160	0.694	23.92	10.49
Laboratory D	1.340	0.574	0	10.92
Laboratory F	1.790	0.754	13.23	25.43
Laboratory G	1.340	0.640	4.73	14.12
			Inter-laboratory CV (%)	
All laboratories	1.380	0.694	17.64	13.26

- The intra- and inter-laboratory reproducibility at 48h and 96h is acceptable.
- The ratio of the highest mean LC50 value to the lowest is 1.43 and 1.40 for 48h and 96h, respectively.
- Comparison of the mean LC50 values at 48h and 96h indicate an increase in toxicity by factor 2.

2,3,6-Trimethylphenol

Analysis of 2,3,6-Trimethylphenol stock solutions and test concentrations

P&G performed analytical measurements of the 2,3,6-Trimethylphenol stock solutions and of the test concentrations for run n° 2 (for details see Annex V).

- There was no difference between the nominal and the measured stock solution concentrations.
- The measured test concentrations were between 92 to 106% of the nominal test concentrations during the 96h exposure.

LC50 values – 2,3,6-Trimethylphenol

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

Table 8: 2,3,6-Trimethyphenol (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the ZFET

2,3,6-Trimethylphenol	Mean LC50 (mg/L)		Intra-laboratory CV (%)	
	48h	**96h**	**48h**	**96h**
Laboratory A	8.91	8.91	14.94	14.94
Laboratory C	10.4	10.3	13.97	13.7
Laboratory D	11.1	11	21.22	22.43
Laboratory F	13.2	13	15.01	13.99
Laboratory G	22.5	22.3	20.44	18.83
			Inter-laboratory CV (%)	
All laboratories	13.2	13.1	40.9	40.88
All laboratories without G	10.9	10.8	16.37	15.77

- The intra-laboratory reproducibility at 48h and 96h is acceptable.
- The inter-laboratory reproducibility is acceptable at 48h and 96h when not considering laboratory G, but beyond the acceptance threshold when considering all laboratories. However, as the biostatistician claims, the 40% CV here is statistically indistinguishable from a much lower CV, including 30%.
- The ratio of the highest mean LC50 value to the lowest is 2.5 for 48h and 96h when evaluating all laboratories. When considering all laboratories other than laboratory G the ratio of highest to lowest LC50 is 1.48 and 1.46 for 48h and 96h, respectively.
- There is no difference in the toxicity at 48h and 96h.

6-Methyl-5-hepten-2-one

Analysis of 6-Methyl-5-hepten-2-one stock solutions and test concentrations

46. P&G performed analytical measurements of the 6-Methyl-5-hepten-2-one stock solutions and of the test concentrations for run n° 2 (for details see Annex V).

- There was no difference between the nominal and the measured stock solution concentrations.
- Substantial losses below 80% of the nominal test concentrations over the 24h renewal period were observed for three concentrations including the highest one (74%).

LC50 values – 6-Methyl-5-hepten-2-one

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

Table 9: 6-Methyl-5-hepten-2-one (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the Zebrafish Embryo Toxicity Test

6-Methyl-5-hepten-2-one	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory B	539	438	41.04	21.68
Laboratory C	277	236	46.64	34.63
Laboratory F	138	137	1.25	2.28
Laboratory G	162	160	5.68	3.61
			Inter-laboratory CV (%)	
All laboratories	279	243	65.85	56.32

- The intra-laboratory reproducibility at 48h and 96h is acceptable for two laboratories (F, G). It is not acceptable for laboratories B and C at 48h (CV>40%). The CVs at 96h are acceptable for laboratory B and for laboratory C above the threshold of 30%.
- The inter-laboratory reproducibility at 48h and 96h is not acceptable.
- The ratio of the highest mean LC50 value to the lowest is 3.3 and 3.2 for 48h and 96h, respectively.
- There is no difference in the toxicity at 48h and 96h.

48. The high variability in the results might be explained by the high volatility of 6-Methyl-5-hepten-2-one (50 times more volatile than ethanol; see Table 1) and possible differences in the handling of the chemical during the preparation of the stock solutions and test concentrations. In addition, none of the laboratories achieved 100% lethality (three achieved at least 50% and one 30% in the highest concentration), indicating that the test concentrations were not appropriate.

Sodium chloride

Analysis of sodium chloride stock solutions and test concentrations

49. P&G performed analytical measurements of their sodium chloride stock solutions and of the test concentrations for run n° 1 (for details see Annex V).

- There was no difference between the nominal and the measured stock solution concentrations.
- The measured test concentrations were between 94.5 to 97.8% of the nominal test concentrations during the 96h exposure.

50. The analytical measurements of the stock solutions performed by the laboratories confirmed the nominal concentration (data not shown).

LC50 values – Sodium chloride

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

Table 10: Sodium chloride (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the ZFET

Sodium chloride	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory B	5040	4490	12.09	7.73
Laboratory C	4370	4270	10.18	6.36
Laboratory F	6530	6390	6.25	8.96
Laboratory G	5420	5420	19.48	19.48
			Inter-laboratory CV (%)	
All laboratories	5340	5140	16.93	18.85

- The intra- and inter-laboratory reproducibility at 48h and 96h is acceptable.
- The ratio of the highest mean LC50 value to the lowest is 1.49 for 48h and 96h.
- There is only a slight difference in the toxicity of sodium chloride at 48h and 96h.

Ethanol

Analysis of ethanol stock solutions and test concentrations

52. P&G performed analytical measurements of the test concentrations for run n° 3 (for details see Annex V).

- The measured test concentrations were between 90.6 to 93.9% of the nominal test concentrations during the 96h exposure.

LC50 values – Ethanol

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

Table 11: Ethanol (3 runs) – mean LC50 values with intra- and inter-laboratory reproducibility of the ZFET

Ethanol	**Mean LC50 (mg/L)**		**Intra-laboratory CV (%)**	
	48h	**96h**	**48h**	**96h**
Laboratory A	13400	11600	15.13	15.71
Laboratory C	12200	12300	14.16	14.14
Laboratory D	13000	12000	7.1	3.08
Laboratory F	12700	11400	7.1	5.5
Laboratory G	14700	12800	0.02	5.1
			Inter-laboratory CV (%)	
All laboratories	13200	12000	7.09	4.78

- The intra- and inter-laboratory reproducibility at 48h and 96h is acceptable.
- The ratio of the highest mean LC50 value to the lowest is 1.2 and 1.04 for 48h and 96h, respectively.
- The mean LC50 values indicate that ethanol is slightly more toxic at 96h.

Overview intra- and inter-laboratory reproducibility

Intra-laboratory reproducibility

54. A summary of the intra-laboratory reproducibility (CV%) calculated based on the mean LC50 is given in Table 12.

Table 12:		Intra-laboratory reproducibility - coefficients of variation for the LC50 values of six chemicals					
		Laboratory (CV%)					
Time	**Chemical**	**A**	**B**	**C**	**D**	**F**	**G**
48h	**Triclosan**	-	2.14	14.15	-	8.68	17.73
	Dibutyl Maleate	10.27	-	23.92	0.00	13.23	4.73
	2,3,6-Trimethylphenol	14.94	-	13.97	21.22	15.01	20.44
	6-Methyl-5-hepten-2-one	-	41.04	46.64	-	1.25	5.68
	Sodium Chloride	-	12.09	10.18	-	6.25	19.48

	Ethanol	15.13	-	14.16	7.10	7.10	0.02
96h	**Triclosan**	-	16.79	37.99	-	5.86	2.60
	Dibutyl Maleate	19.16	-	10.49	10.92	25.43	14.12
	2,3,6-Trimethylphenol	14.94	-	13.70	22.43	13.99	18.83
	6-Methyl-5-hepten-2-one	-	21.68	34.63	-	2.28	3.61
	Sodium Chloride	-	7.73	6.36	-	8.96	19.48
	Ethanol	15.71	-	14.14	3.08	5.50	5.10

- : chemical not tested in the given laboratory (see also Table 5)

- At 48h, the overall intra-laboratory reproducibility is acceptable regardless of the chemicals tested with 25 CV values ranging from 0-24% with the exception of one chemical (6-Methyl-5-hepten-2-one) where two CVs are >40%.
- At 96h, the overall intra-laboratory reproducibility is acceptable regardless of the chemicals tested with 25 CV values ranging from 0-26% with the exception of two chemicals (6-Methyl-5-hepten-2-one, Triclosan) where two CVs are >34%.

Inter-laboratory reproducibility

55. A summary of the inter-laboratory reproducibility (CV%) calculated based on the mean LC50 is given in Table 13.

Table 13: Inter-laboratory reproducibility - coefficients of variation for the LC50 values of six chemicals

Time	Chemicals	CV (%)	N
48h	**Triclosan**	9.24	4
	Dibutyl Maleate	17.64	5
	2,3,6-Trimethylphenol	40.90 (16.37)*	5
	6-Methyl-5-hepten-2-one	65.85	4
	Sodium Chloride	16.93	4
	Ethanol	7.09	5
96h	**Triclosan**	11.80	4
	Dibutyl Maleate	13.26	5
	2,3,6-Trimethylphenol	40.88 (15.77)*	5
	6-Methyl-5-hepten-2-one	56.32	4
	Sodium Chloride	18.85	4
	Ethanol	4.78	5

N: number of laboratories that tested the chemical;
****: without laboratory G***

- For four chemicals, the inter-laboratory reproducibility is acceptable for both time points.

- For 2,3,6-Trimethylphenol the inter-laboratory reproducibility is with a CV>40% beyond the acceptance threshold. Without considering laboratory G, the inter-laboratory reproducibility is acceptable and lies in the range of the other four chemicals.
- The inter-laboratory reproducibility for 6-Methyl-5-hepten-2-one is not acceptable. The high variability of the results might be due to the high volatility of the chemical.

Conclusions Phase 1b

56. The VMG concludes that the ZFET test was successfully transferred from the lead laboratory to the participating laboratories.

57. The intra- and inter-laboratory reproducibility of the LC50 values is promising: for five chemicals it is very good, however, reproducibility is lower for the volatile chemical 6-Methyl-5-hepten-2-one. In this context, it is advisable to establish guidance for testing of volatile chemicals since there two laboratories have a rather low toxicity of 6-Methyl-5-hepten-2-one, which might indicate that the chemical evaporated during the handling of the chemical, e.g. preparation of the stock solution, test concentrations etc.

58. Analytical measurements confirm exposed test concentrations ≥80% except for 2 chemicals (Dibutyl maleate and 6-Methyl-5-hepten-2-one).

59. There were slight differences in lethality at 48h and 96h for very toxic chemicals (factor 1.5 to 2). This is expected for compounds with higher hydrophobicities which would take longer to traverse biological membranes.

COMPARISON OF ZFET AND FISH LC50 VALUES

60. 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 14 is meant to give a preliminary idea of the predictive capacity of the ZFET test for acute fish toxicity.

Table 14: Comparison of ZFET LC50 values and the 96h acute fish LC50 values

Phase 1 chemicals	**ZFET mean LC50 (mg/L)**		**Fish acute* mean LC50 (mg/L)**	**Ratio Fish LC50:FET LC50**
	48h	**96h**	**96h**	**96h**
Triclosan	0.42	0.3 (4) **	0.5 (2)**	1.67
Dibutyl maleate	1.38	0.7 (5)	1.2 (1)	1.71
2,3,6-Trimethylphenol	10.9***	10.8***(4)	8.2 (1)	0.75

3,4-Dichloroaniline	3.2	2.7 (5)	8.59 (1)	3.18
6-Methyl-5-hepten-2-one	279	243 (4)	85.7 (1)	0.35
Sodium chloride	5340	5140 (4)	7700 (14)	1.49
Ethanol	13200	12000 (5)	14200 (1)	1.18

* Measured fish LC50 values were retrieved from literature and the OECD QSAR toolbox (Version 2.0). Fish species: fathead minnow, rainbow trout (for dibutyl maleate) and zebrafish (for 3,4 Dichloroaniline); flow-through or semi-static test set-up
** indicates the number of LC50 values used to calculate the mean;
*** without laboratory G

61. The comparison reveals that:

- the two chemicals non-toxic to fish are also non-toxic in the ZFET;
- the moderately fish toxic chemical 6-methyl-5-hepten-2-one was non-toxic in the ZFET; and
- the four other toxic chemicals showed toxicities in same order of magnitude.
- the ZFET was slightly more sensitive on average in 5 of 7 cases although some differences are likely not biologically or statistically significant.

62. The limited number of chemicals tested does not allow a sound conclusion on the predictive capacity of the ZFET. In Phase 2, an additional 13 chemicals will be tested covering a wide range of toxicities, modes of action and chemical categories.

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DIN (2001) German standard methods for the examination of water, waste water and sludge – Subanimal testing (group T) – Part 6: Toxicity to fish. Determination of the non-acute poisonous effect of waste water to fish eggs by dilution limits (T 6). DIN 38415-6; German Standardization Organization.

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T. (2009) Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comparative Biochemistry and Physiology, Part C, Toxicol Pharmacol -149 (2), 196-209.

OECD (1992) Test Guideline 203. OECD Guideline for Testing of Chemicals. Fish, Acute Toxicity Test. Available:
[http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html].

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ANNEX I - STUDY DOCUMENTS AND METHOD DESCRIPTION

1. Documents

For Phase 1a and 1b, the following documents were agreed upon by the Validation Management Group (VMG) and distributed to the laboratories by the coordinator:

Phase 1a – Single run with 3,4-Dichloroaniline
- Trial Plan - TP_ZFET_OECD_1a_V01.6
- Standard Operating Procedure - SOP_ZFET_OECD_V02.7
- RT_ZFET_OECD_1a_V01.2

Phase 1a – Three runs with 3,4- Dichloroaniline
- Trial Plan - TP_ZFET_OECD_1a_V01.7
- Standard Operating Procedure - SOP_ZFET_OECD_V02.8
- RT_ZFET_OECD_1a_V01.3

Phase 1b – Testing of 6 chemicals
- Trial Plan - TP_ZFET_OECD_1b_V01.1
- Standard Operating Procedure - SOP_ZFET_OECD_V02.9
- RT_ZFET_OECD_1b_V01.1

These documents are available on request.

2. Brief description of the Zebrafish Embryo Toxicity Test based on the above SOPs:

Newly fertilised zebrafish eggs (20 per test concentration and control) were exposed for 96h to 5 concentrations of one chemical and the appropriate controls dilution water (negative control), solvent control and positive control.

Zebrafish:
- A breeding stock of unexposed and healthy mature zebrafish *Danio rerio* with an age between 4 and 18 months was used by the laboratories for the egg production.

Dilution water
- Dilution water is prepared according to OECD TG 203 (OECD, 1992).

Zebrafish egg production
- Eggs are produced via spawning groups or mass spawning.

Method

- The 24-well plates and glass vessels were pre-saturated with the respective concentrations of the chemicals and controls for at least 24h before the day of the test. (Note: not performed for the *"Single Run with 3,4-DCA")*.

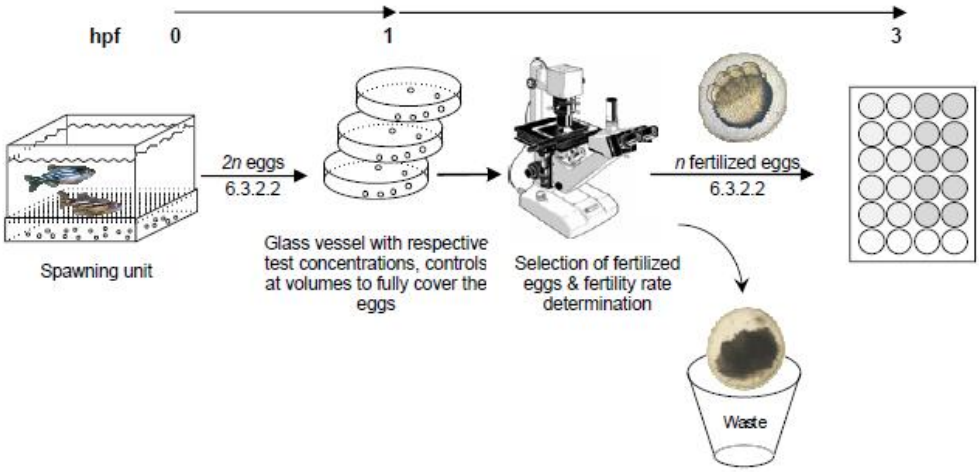


Fig. 1: Scheme of the ZFET test procedure (from left to right): collection of the eggs, pre-exposure to respective test concentrations/controls in glass vessels immediately after fertilisation, selection of fertilised eggs with an inverted microscope or binocular and distribution of fertilised eggs into prepared 24-well plates, n = number of eggs required for the test run (kindly provided by University of Heidelberg and modified for the study).

- After the selection step, the fertilised eggs were transferred into 24-well plates covered with self-adhesive foil or lids provided with plates and incubated at 26 ± 1 °C for 96h. Control of the light cycle to 14h light and 10h dark is achieved by keeping the eggs in either an incubator or separate room equipped with an automatic light control.
- Renewal of the test concentrations and the negative control was daily performed with freshly prepared test concentrations from the stock solution (Note: not performed for the *"Single Run with 3,4-DCA")*.
- Measurements of test conditions such as dissolved oxygen concentration, pH, total hardness, temperature and conductivity were performed for the controls and the highest concentration.

Recording of toxicity

Four apical endpoints were recorded daily as indicators of acute lethality in fish:

- coagulation of embryo
- lack of somite formation
- non-detachment of tail bud from the yolk sac
- lack of heart-beat

In addition to the four apical endpoints, hatching rate is daily recorded since non-hatching may represent an important toxic effect knowing that zebrafish embryos usually hatch after 72h.

Acceptance Criteria

For a run to be considered qualified the following criteria were applied:

- The fertility rate of the parent generation should be ≥70%.
- The dissolved oxygen concentration should be ≥80 % of the air saturation value at the beginning of the test.
- The water temperature should be maintained at 26 ± 1 °C in test chambers at any time during the test.
- Overall survival of embryos in the negative external control and, where relevant, in the solvent control should be ≥90% until the end of exposure.
- Exposure to the positive control (e.g. 4.0 mg/l 3,4-dichloroaniline) should result in a minimum mortality of 30 % (Note: this was only performed for phase 1b).
- Controls and test solutions must be renewed on a daily basis (Note: not performed for the *"Single Run with 3,4-DCA"*).

ANNEX II - ANALYSIS OF 3,4-DCA CONCENTRATIONS IN FET STOCK AND EXPOSURE SOLUTIONS

1. Introduction

Analytical verification of 3,4-DCA in aqueous stock and exposure solutions utilized during an international validation study of the FET was performed by the Trace Analytical Group, Procter & Gamble, Mason Business Center, Cincinnati, Ohio USA. 3,4-DCA is proposed for use as an internal positive control test chemical in the FET (*Zebrafish Embryo Toxicity Test*, Standard Operation Procedure, SOP ZFET OECD V02.7, April 28th, 2009). For the purposes of this study stock solutions would ideally be stable as a single preparation for several weeks to months resulting in individual stock solutions to be usable for extended time periods. Also, exposure solutions in multi-well plates would be stable up to 96 hr (maximum duration of a test) which would also minimize potential for exposures to contribute towards variable FET results.

The objectives of this study were to:

- Determine the appropriate duration for the holding of 3,4-DCA stock solutions in aquatic toxicity studies;
- Verify stock solution concentrations by participating laboratories in the OECD validation program for the FET; and,
- Determine exposure concentrations in one representative laboratory (P&G).

2. Methods

Preparation of Stock Solutions

Seven laboratories participated in this exercise:

Name	**Contact**
University of Heidelberg, Germany	Thomas Braunbeck
Procter & Gamble, USA	Scott Belanger
IVM, The Netherlands	Juliette Legler
UFZ, Germany	Stefan Scholz
RIVM, The Netherlands	Leo van der Ven
VITO, Belgium	Hilda Witters
Institute of Industrial Chemistry, Poland	Przemyslaw Fochtman

Stock solution preparation was outlined in the Phase 1a Trial Plan (Zebrafish Embryo Toxicity Test, Evaluation of transferability, intra- and inter-laboratory reproducibility Trial Plan for Phase 1a – Transferability, *TP_ZFET_OECD_1a_V01.6*, April 28th 2009) and is also given below:

- 50 mg 3,4-DCA was dissolved in 500 mL of FET dilution water
- Solutions were stirred in a light-proof vessel for 24 hr at room temperature
- pH was adjusted to ± 0.5 of the dilution water
- Stocks were kept refrigerated (1-8° C) in the dark
- Stocks were refrigerated and before use were stirred and brought to room temperature for at least 30 minutes

Shipping of Samples

Samples of stock solutions (10-20 mL) were taken by researchers and sent in borosilicate amber glass bottles (VWR Catalogue 80076-572 or similar). The samples were then shipped by participating laboratories following instructions given in the Trial Plan to P&G where they were received by the laboratory of K. R. Wehmeyer. Shipments were successfully received at P&G by only 4 of 7 laboratories due to a variety of export difficulties imposed by qualified shippers in Europe at the point of export. These were University of Heidelberg, VITO, The Institute of Industrial Organic Chemistry and Procter & Gamble. The nature of rejection of exportation of samples was not consistent and appeared somewhat random. Future shipments of additional chemical stock solutions will explore alternative strategies.

Stock and Exposure Solution Analyses

Stock solutions were analyzed several times from approximately late April through late September, 2009 to assess long-term stability of stocks.
In addition to stock solutions from the above listed laboratories, exposure solutions were evaluated from the P&G test on two occasions.

1. In the first study (intra-laboratory transferability investigation), exposures were not renewed and the test was run as a static exposure. A set of surrogate multi-well plates were used for the purpose of analytical verification of exposure. Each vial was pre-rinsed with exposure solution. Samples (1 mL) were taken from surrogate multi-well plates and immediately sent to the analytical laboratory. Sampling was performed in triplicate to gain a better understanding of well-to-well and temporal variability. Exposure concentrations were 0 (dilution water), 0.5., 1, 2, 3.7, 4, and 8 mg/L. The 3.7 mg/L exposure corresponded with the previously proposed internal control exposure concentration (ZFET, Standard Operation Procedure, SOP ZFET OECD V02.7, April 28th, 2009) whose adequacy was being verified in this study.

2. In the second study (intra-laboratory variability study), exposures were renewed daily (same nominal exposure concentrations as above) following initial soaking of wells using the appropriately diluted exposure concentration. Solutions were renewed at 24, 48, and 72 hr. Samples from representative exposure wells were taken before and after renewal (4 times each).

Analytical Methods

Analytical methods applied to the stock solutions were evaluated by an HPLC-UV method with a C18 column on a Waters Alliance HPLC with a Waters PDA 996 Detector. Exposure solutions were analyzed by reverse-phase HPLC-MS/MS with a C18 column on a Sciex API 3000 triple quadrupole mass spectrometer in positive ion electrospray ionization mode.

3. Results

Stock Solutions

Analysis of stock solutions indicated that all laboratories successfully prepared 100 mg/L solutions for use in FET exposures (Table 1). Repeat analysis of solutions held for 4 months after preparation under refrigerated conditions suggests that concentrations declined over this period but universally remained at >90% of the initial nominal concentration. Details of individual sample Quality Control samples and individual RSDs of stock solutions are provided under request (M. J. Karb memo to J. Rawlings, S.E. Belanger, and P. Sun, dated 23 July 2009). Standard curves, RSD, and quality control checks all indicate the appropriateness of both the method and interpretation of the results. Detection limits for these analyses ranged from 0.001-0.002 mg/L.

Exposure Solutions: Static Test

Exposure solutions were highly repeatable and all were approximately 80% of nominal (Table 2). Declines in exposure concentration occurred at all concentrations through the test (Figure 1). Replicate wells were highly repeatable with overall Coefficients of Variation (CV) across all treatments ranging from 1.9-5.7% (Data in Memo from M. Karb, 17 July 2009). Control solutions were universally below the detection limit of 0.002 mg/L.

Exposure Solutions: Static Renewal Test

Because exposure concentrations appeared to decline through time and measured concentrations were around the recovery level normally used to indicate static renewal or flow through tests designs would be useful (OECD TG 203) the Trial Plan was modified to accommodate a static renewal design (Zebrafish Embryo Toxicity Test, Evaluation of transferability, intra- and inter-laboratory reproducibility Trial Plan for Phase 1a – Transferability, *TP_ZFET_OECD_1a_V01.7*, June 17th 2009). The overall results were remarkably similar to that of the static only design (Table 3) as well as for the declines in exposure concentration at all concentrations through the test (Figure 2). Coefficients of variation ranged from 0.9-9.9% across all exposure concentrations. Arithmetic and geometric averaging methods are candidates for use in expressing average exposure conditions for toxicity data interpretation and were compared as a final exercise. Both methods yielded similar results (Table 4) are in these experiments either would be equally suitable.

4. Conclusions

- Analyses confirm that 3,4-DCA stock solutions were successfully produced by 4 of 7 laboratories (shipment of samples by 3 of the 7 laboratories were stopped by certified exporters for unknown reasons). Inter-laboratory differences are relatively small and would have little impact on execution of dilution series across laboratories. It is highly likely that confirmation of exposures in the tests themselves would also be similar.

- Stock solutions declined through time. It is recommended that stock solutions be used for no more than 2 to 3 months if kept in the dark under refrigerated conditions.
- Measurement of 3,4-DCA concentrations in multi-well plates during FET exposures were determined under static and static renewal conditions. Results were highly similar with measured concentrations being approximately 80% of nominal. Under static conditions, concentrations declined somewhat through time. Measurements were highly repeatable within treatments.
- Correction of LC50 determinations based on measured exposures would not be required under conditions outlined in current environmental toxicity test guidelines (e.g. OECD TG 203), but in any case actual LC50 or EC50 calculations would be lower by virtue of the measurements made.

Table 1. Overview of the analysis of stock solutions (all 100 mg/L nominal) from 4 participating laboratories.

Laboratory	Date Stock Solution was Prepared	Date Analyzed	Sample	Value (mg/L)
P&G, USA	1 May 2009	11 May 2009	1	98.0
			2	98.0
		23 July 2009	1	93.9
			2	93.0
			3	94.7
			4	94.5
Heidelberg University, Germany	30 Apr 2009	27 May 2009	1	106.2
			2	106.4
		28 Sept 2009	2	100.0
Institute of Industrial Chemistry, Poland	15 May 2009	27 May 2009	1	103.0
			2	103.3
		28 Sept 2009	2	97.0
VITO, Belgium	11 May 2009	28 May 2009	1	104.7
			2	104.9
		28 Sept 2009	1	100.5

Table 2: Analytical exposure verification results of the static FET conducted at P&G (4-8 May 2009). A total of 5 samples (0, 24, 48, 72, and 96 hr) from triplicate surrogate wells were used per test concentration. The 8 mg/L exposure concentration was terminated after 24 hr due to 100% mortality.

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	SD (mg/L)	Mean % of Nominal	SD % of Nominal
0	BQL	BQL	N/A	N/A
0.5	0.38	0.01	76.2	1.6
1	0.77	0.04	77.0	4.3
2	1.64	0.04	81.8	2.1
4	3.32	0.09	82.9	2.2
8	6.87	0.25	85.9	3.1
3.7	2.97	0.17	80.1	4.6

SD: Standard Deviation; BQL: Below Quantifiable Limit; N/A: Not Applicable

Table 3: Analytical exposure verification results of the static renewal FET conducted at P&G (12-16 July 2009). A total of 8 samples (0, 24, 48, 72, and 96 hr at beginning and end of renewals) from triplicate surrogate wells (1ml) were used per test concentration. The 8 mg/L exposure concentration was terminated after 24 hr due to 100% mortality.

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)			Mean % of Nominal		
	Old	New	Overall (Old and New Combined)	Old	New	Overall (Old and New Combined)
0	BQL	BQL	BQL	N/A	N/A	N/A
0.5	0.43	0.46	0.44	85.8	91.9	88.5
1	0.81	0.83	0.82	80.9	83.4	82.1
2	1.62	1.65	1.63	80.3	82.6	81.3
4	3.26	3.24	3.25	81.5	81.1	81.3
8	5.18	5.10	5.15	64.8	63.8	64.4
3.7	2.89	2.93	2.90	78.0	79.1	78.5

BQL: Below Quantifiable Limit; N/A: Not Applicable

Table 4: Comparison of mean concentrations based upon arithmetic versus geometric averaging methods.

Nominal Concentration (mg/L)	Mean Measured Concentration - Arithmetic (mg/L)	Mean Measured Concentration - Geometric (mg/L)	Mean Measured Concentration - Arithmetic (mg/L)	Mean Measured Concentration - Geometric (mg/L)
	Static Test		Static Renewal Test	
0	BQL	BQL	N/A	N/A
0.5	0.38	0.38	0.44	0.44
1	0.77	0.77	0.82	0.82
2	1.64	1.63	1.63	1.63
4	3.32	3.31	3.25	3.24
8	6.87	6.87	5.15	5.15
3.7	2.97	2.96	2.90	2.90

BQL: Below Quantifiable Limit; N/A: Not Applicable

Figure 1: Exposure data for a static FET on 3,4-DCA conducted at P&G. Vertical bars indicate 1 SD of the mean.

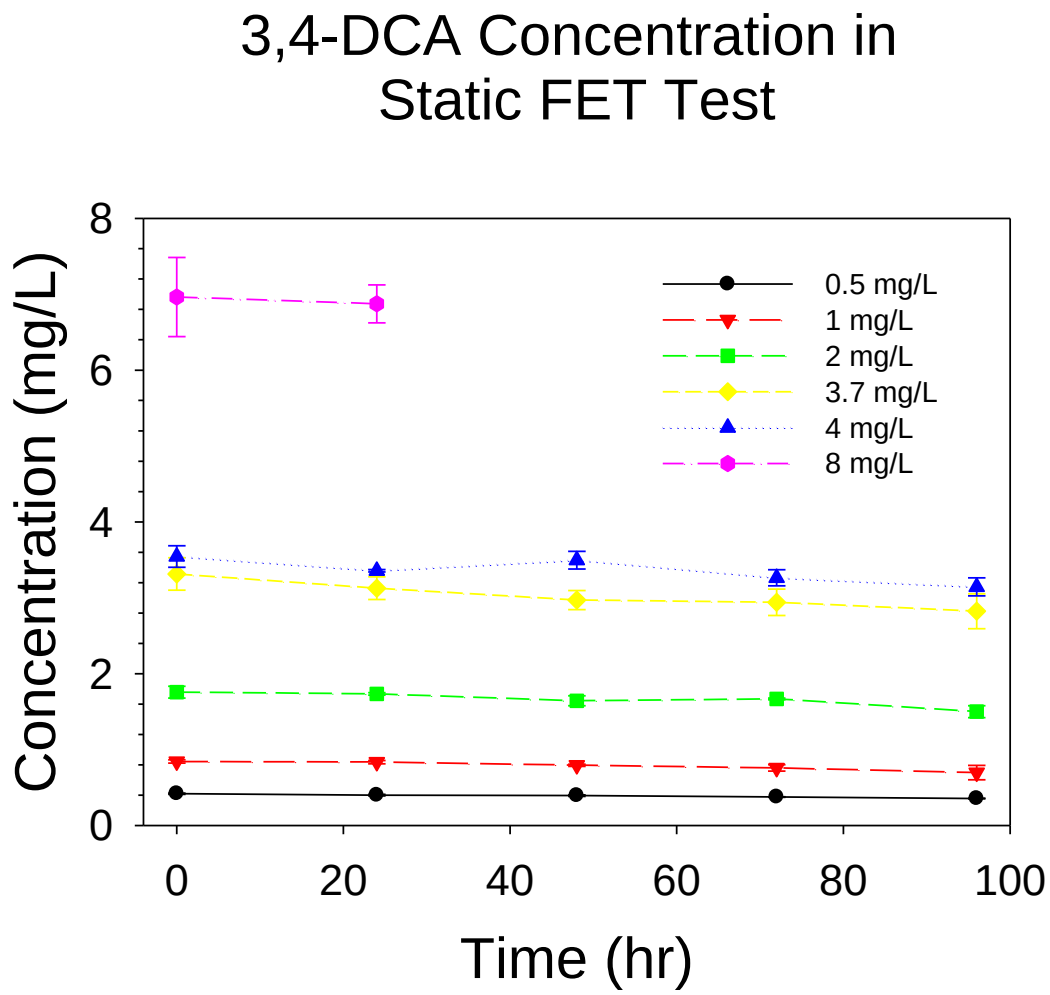
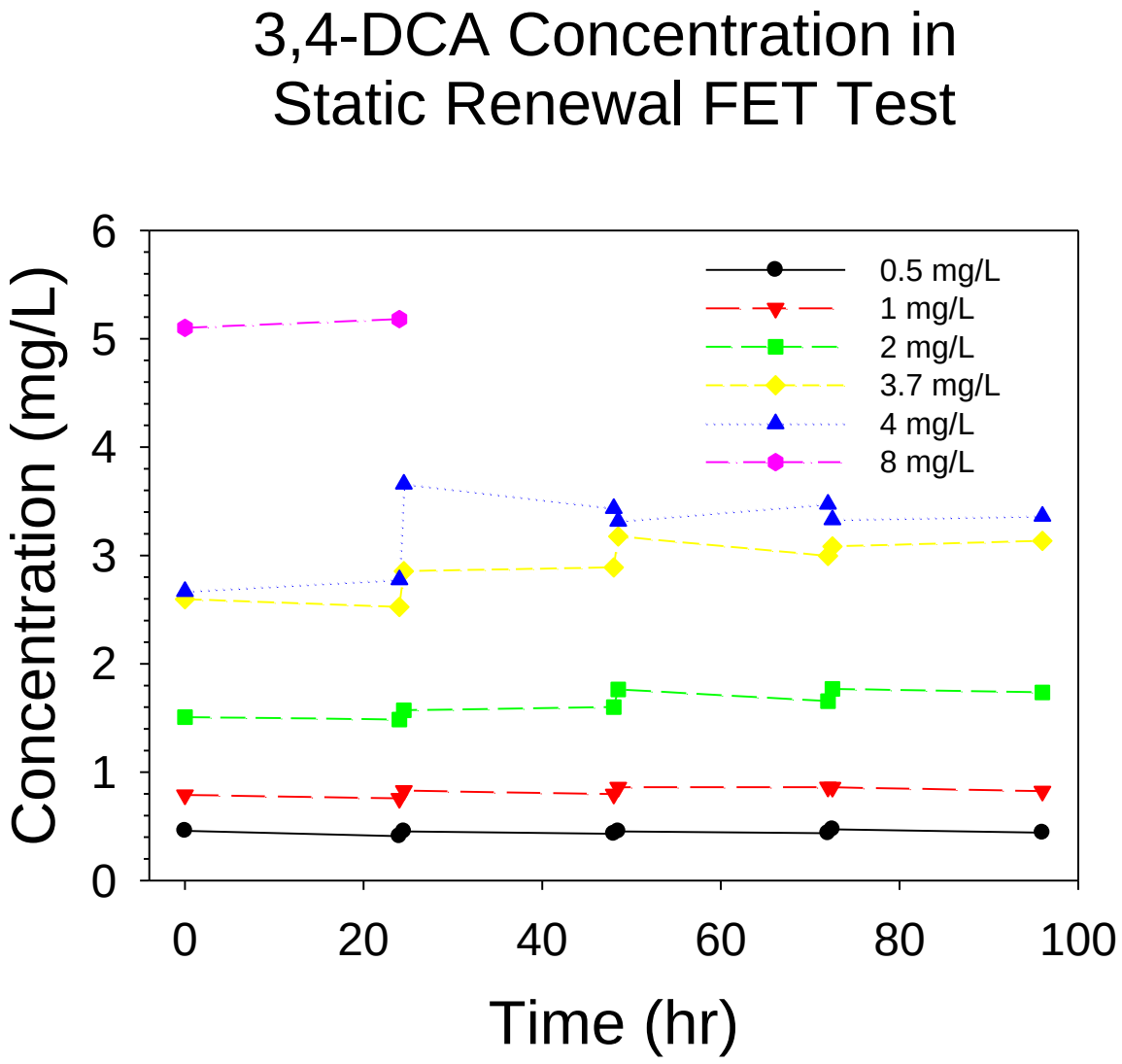


Figure 2: Exposure data for a static-renewal FET on 3,4-DCA conducted at P&G. Vertical bars indicate 1 SD of the mean



ANNEX III - STATISTICAL REPORT PHASE 1A: SINGLE RUN WITH 3,4-DCA

Authors:
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JRC/IHCP/ECVAM, Ispra, ITALY

1. Data

Data were received on 17 June 2009 from François Busquet via e-mail.

2. Process of data analysis

- 2 models were used: log logistic regression with LC50 (*) or log(LC50) (**) as parameter and asymptotic 95% confidence intervals (Hill-model with the lower limit at 0 and the upper limit at 1) as recommended by the OECD Series on Testing and Assessment No. 54 [1].

  * Two parameters: LC50 and the slope (*b*)

  $$y = \frac{1}{1+\exp\left[b\left(\log(x)-\log(\mathrm{LC50})\right)\right]}$$

  ** Two parameters: log(LC50) and the slope (*b*)

  $$y = \frac{1}{1+\exp\left[b\left(\log(x)-\mathrm{LC50}\right)\right]}$$

- Confidence intervals were calculated using the delta method and the t-distribution as described elsewhere [2-4]. The calculations were performed with R 2.9.2 and the package drc_1.7-7 with the functions drm() and ED().
- For “all” the data were collected in one dataset.
- The background mortality (negative controls) was not taken into account, as it does not provide any information for the two-parameter log-logistic regression model [1].
- Under the assumption that the background mortality is about 2% in the current study, the bias that will be introduced because of leaving out the background mortality parameter in the log-logistic regression model is negligible and has the advantage of a more robust model in general.

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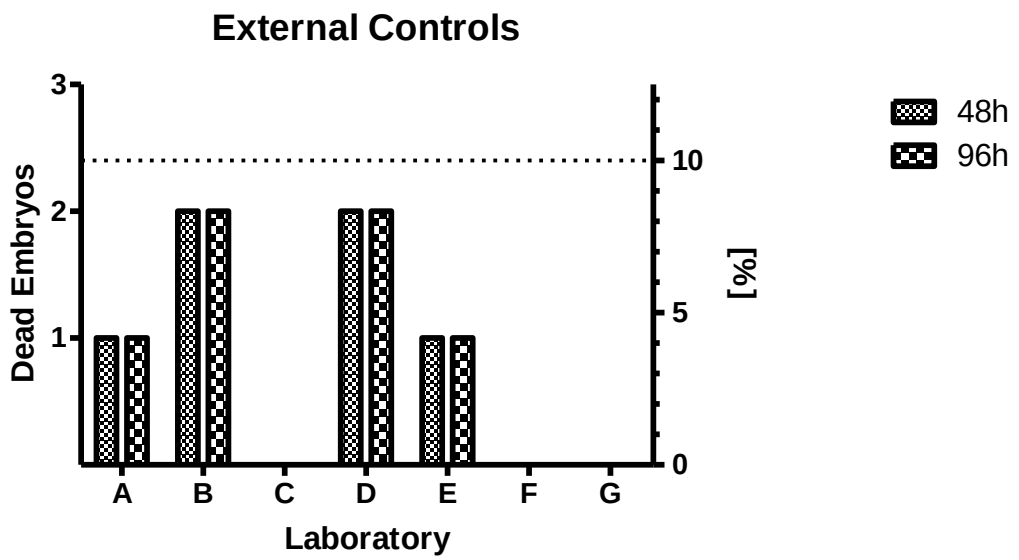
[1].OECD Series on Testing and Assessment No. 54: Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to application. Chapter 6.2, p 63ff

[2] van der Vaart, A. W. (1998): Asymptotic Statistics. Cambridge University Press, Cambridge. Chapter 3
[3] Weisberg, S. (2005): Applied Linear Regression. John Wiley and Sons, New York, third edition. pp. 120-122
[4] Ritz C, Streibing JC. (2008): Nonlinear Regression with R. Springer, New York. Chapter 7.4

3. Results

3.1 Controls



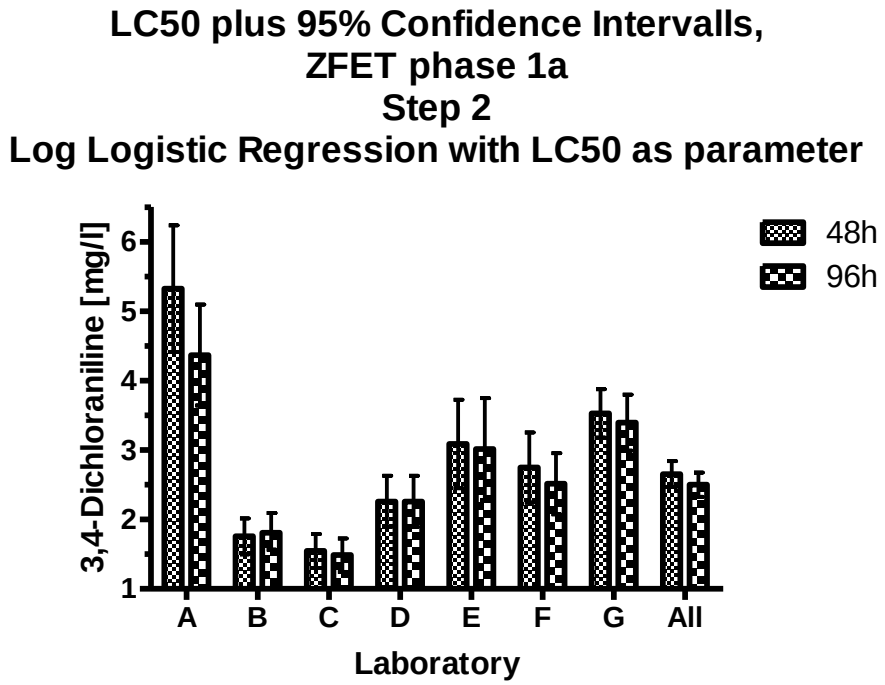
3.2 Summary of LC50 of single run with 3,4-Dichloroaniline

48h	Log-logistic with LC50 as parameter				Log-logistic with log(LC50) as parameter			
Lab	LC50 [mg/l]	95%CI -	95%CI +	Model fit	LC50 [mg/l]	95%CI -	95%CI +	Model fit
A	5.3	4.4	6.2	not reliable *	5.3	4.5	6.3	not reliable*
B	1.8	1.5	2.0	ok	1.8	1.5	2.0	ok
C	1.5	1.3	1.8	ok	1.5	1.3	1.8	ok
D	2.3	1.9	2.6	not reliable **	2.3	1.9	2.7	not reliable **
E	3.1	2.4	3.7	ok	3.1	2.5	3.8	ok
F	2.7	2.2	3.3	ok	2.7	2.3	3.3	ok
G	3.5	3.2	3.9	ok	3.5	3.2	3.9	ok
All	2.7	2.5	2.8	ok	2.7	2.5	2.8	ok

96h	Log-logistic with LC50 as parameter				Log-logistic with log(LC50) as parameter			
Lab	LC50 [mg/l]	95%CI -	95%CI +	Model fit	LC50 [mg/l]	95%CI -	95%CI +	Model fit
A	4.4	3.6	5.1	not reliable *	4.4	3.7	5.2	not reliable *
B	1.8	1.6	2.1	ok	1.8	1.6	2.1	ok
C	1.5	1.2	1.7	ok	1.5	1.3	1.7	ok
D	2.3	1.9	2.6	not reliable **	2.3	1.9	2.7	not reliable **
E	3.0	2.3	3.7	ok	3.0	2.4	3.8	ok
F	2.5	2.1	3.0	ok	2.5	2.1	3.0	ok
G	3.4	3.0	3.8	ok	3.4	3.0	3.8	ok
All	2.5	2.3	2.7	ok	2.5	2.3	2.7	ok

***** The LC50 estimate cannot be considered as reliable, since in Laboratory A only the highest concentration induced high mortality. For a reliable LC50 estimate at least one concentration with an intermediate toxicity response would be necessary.

** The LC50 estimate for Laboratory D cannot be considered reliable since there is a bad curve fitting.



"Step 2" refers to "Single Run with 3,4-DCA"

Individually fitted models/curves

Individually fitted models/curves are shown on the following pages for the log logistic regression with LC50 as parameter.

Figure 1a: Laboratory A - single run with 3,4-Dichloroaniline – 48h

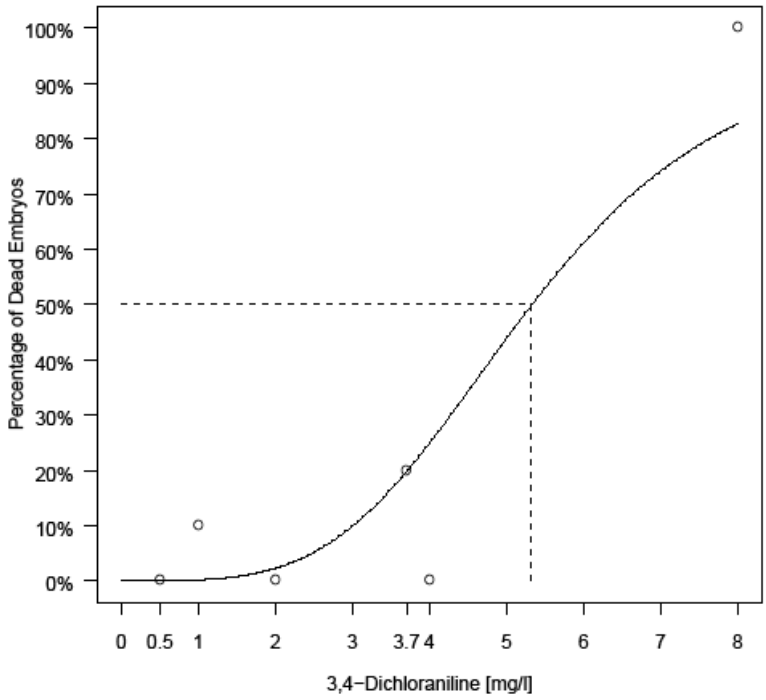


Figure 1b: Laboratory A - single run with 3,4-Dichloroaniline – 96h

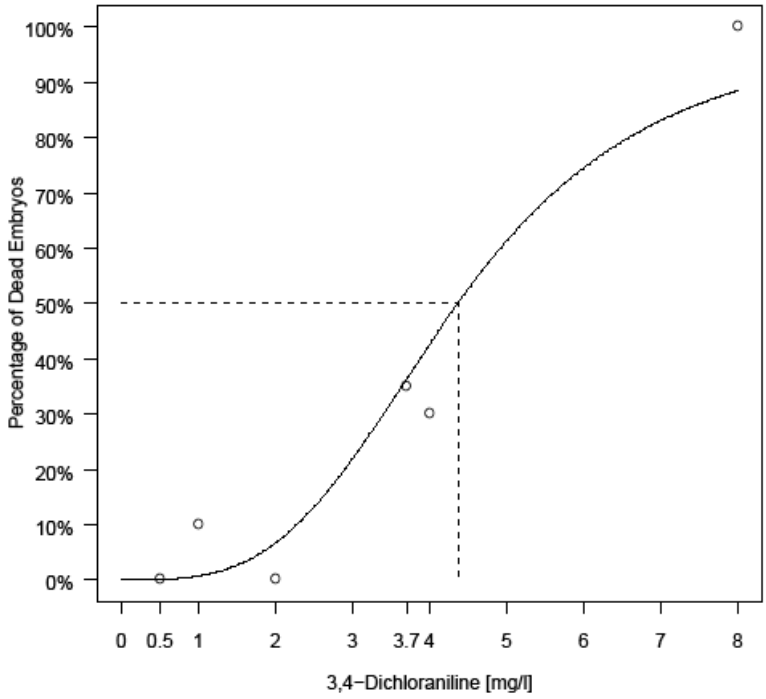


Figure 2a: Laboratory B - single run with 3,4-Dichloroaniline – 48h

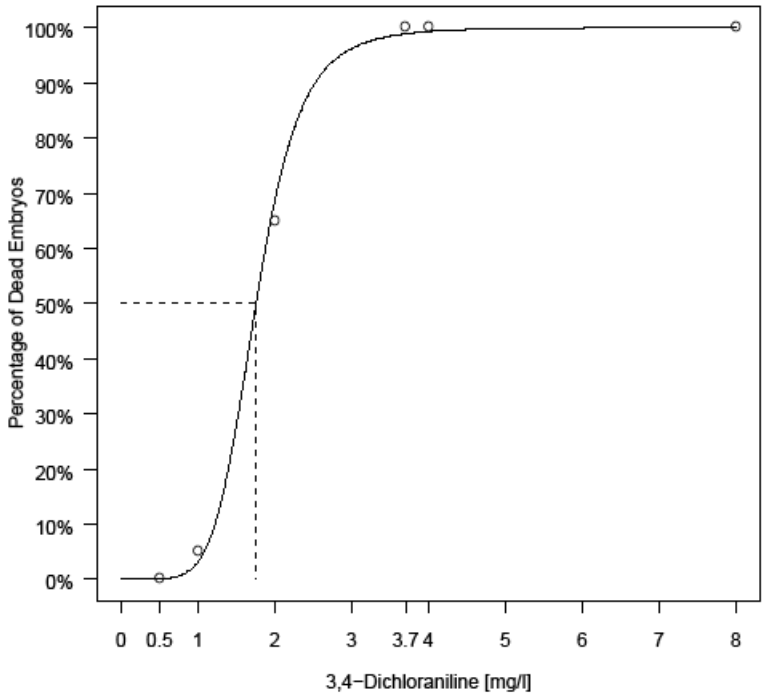


Figure 2b: Laboratory B - single run with 3,4-Dichloroaniline – 96h

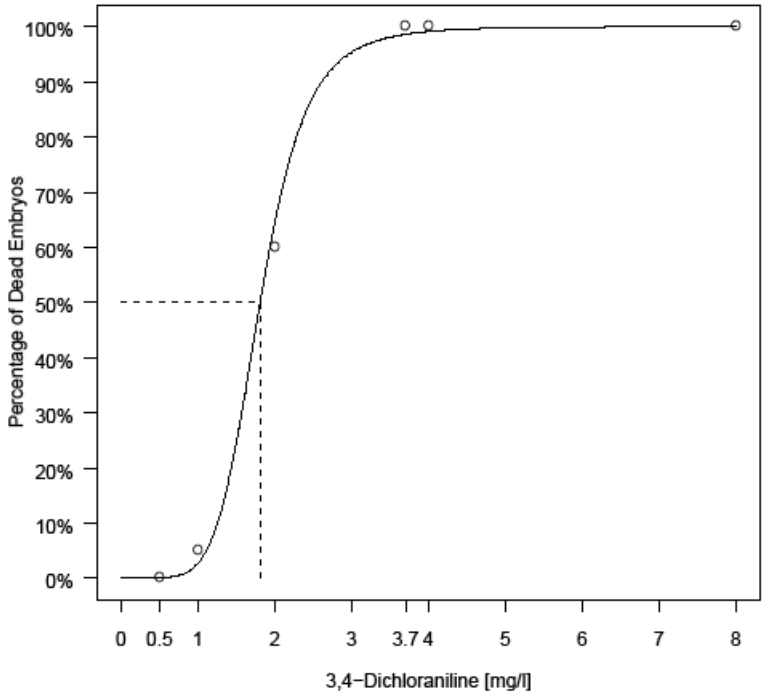


Figure 3a: Laboratory C - single run with 3,4-Dichloroaniline – 48h

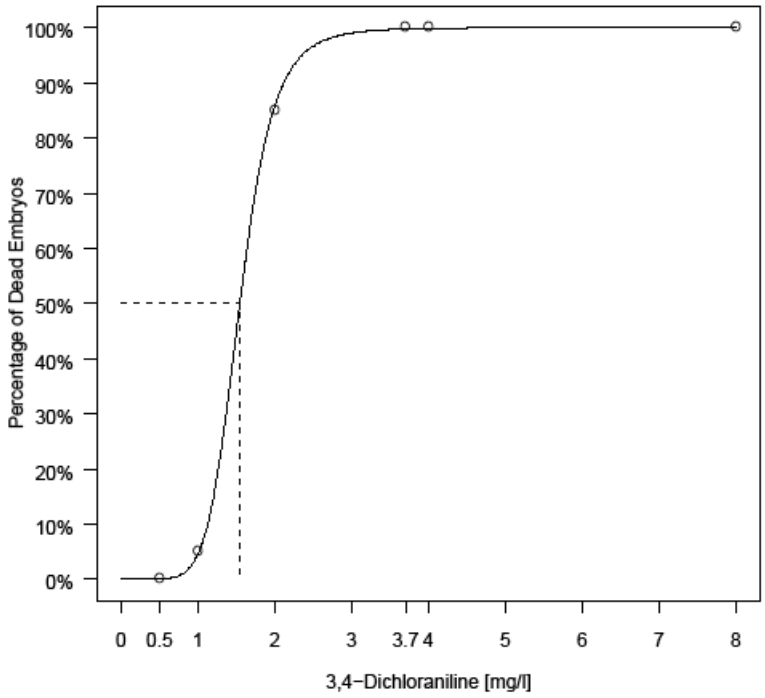


Figure 3b: Laboratory C - single run with 3,4-Dichloroaniline – 96h

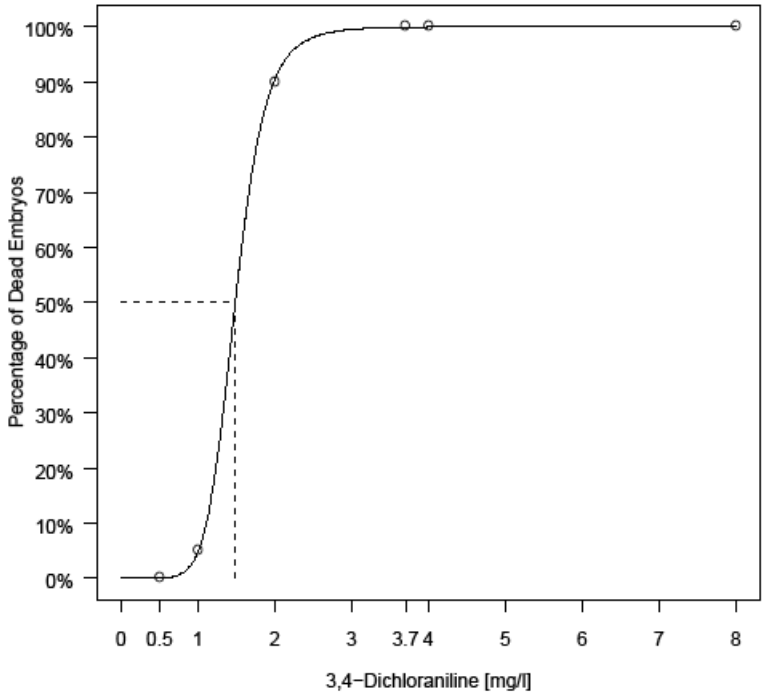


Figure 4a: Laboratory D - single run with 3,4-Dichloroaniline – 48h

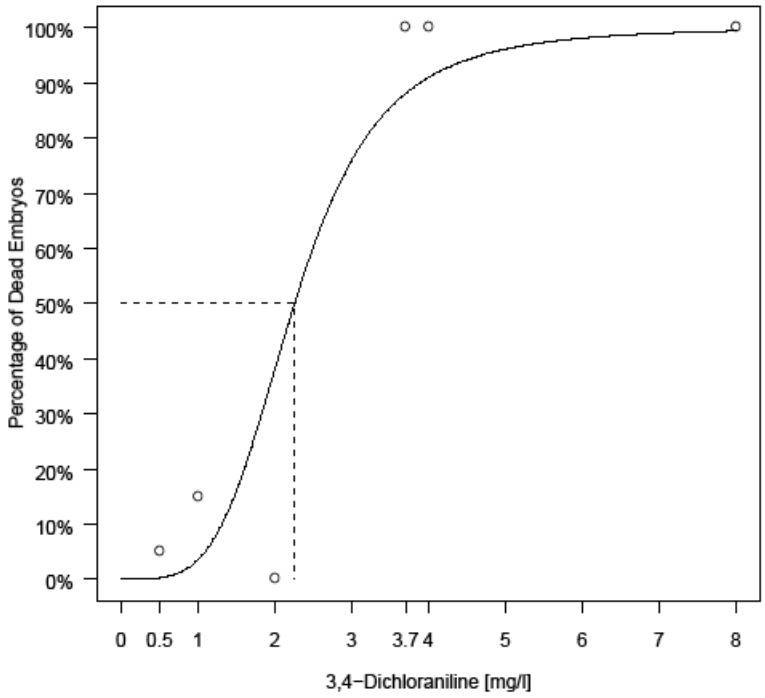


Figure 4b: Laboratory D - single run with 3,4-Dichloroaniline – 96h

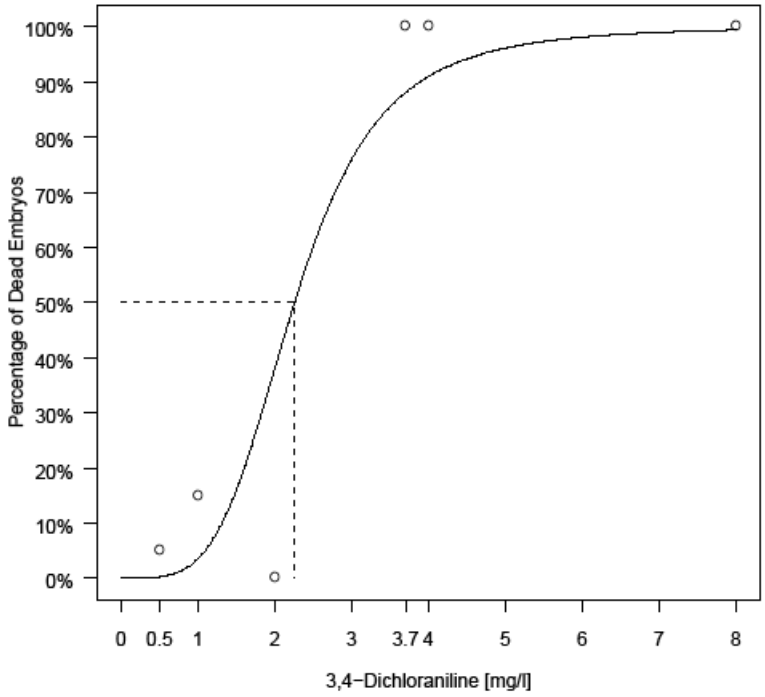


Figure 5a: Laboratory E - single run with 3,4-Dichloroaniline – 48h

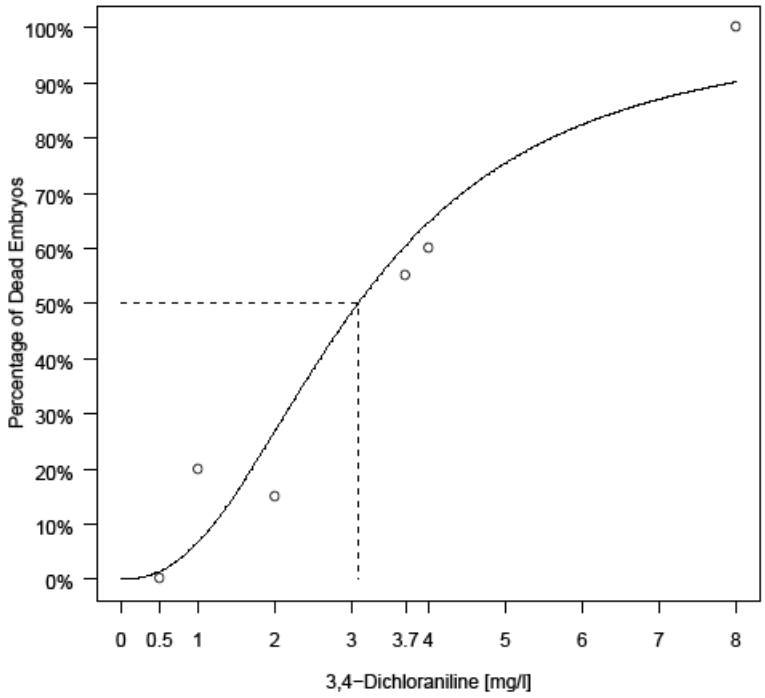


Figure 5b: Laboratory E - single run with 3,4-Dichloroaniline – 96h

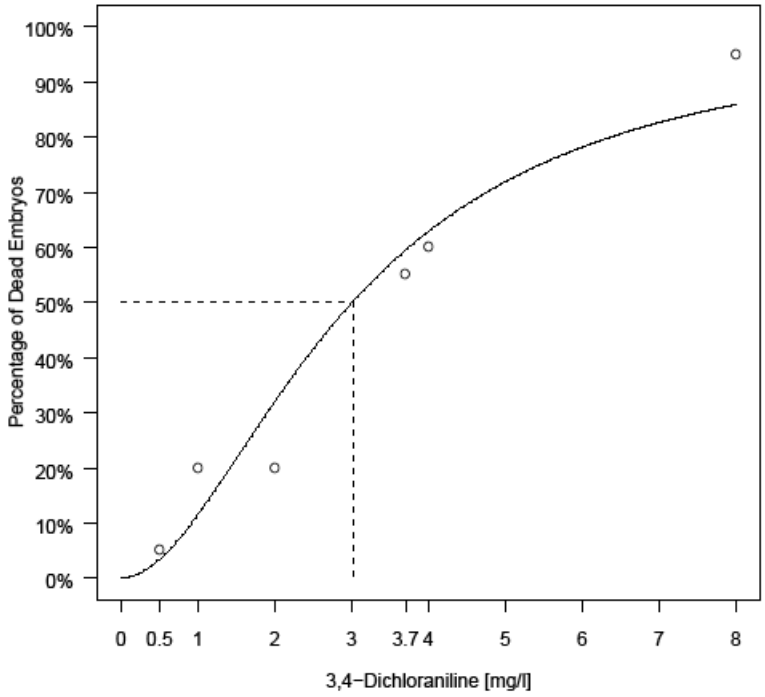


Figure 6a: Laboratory F - single run with 3,4-Dichloroaniline – 48h

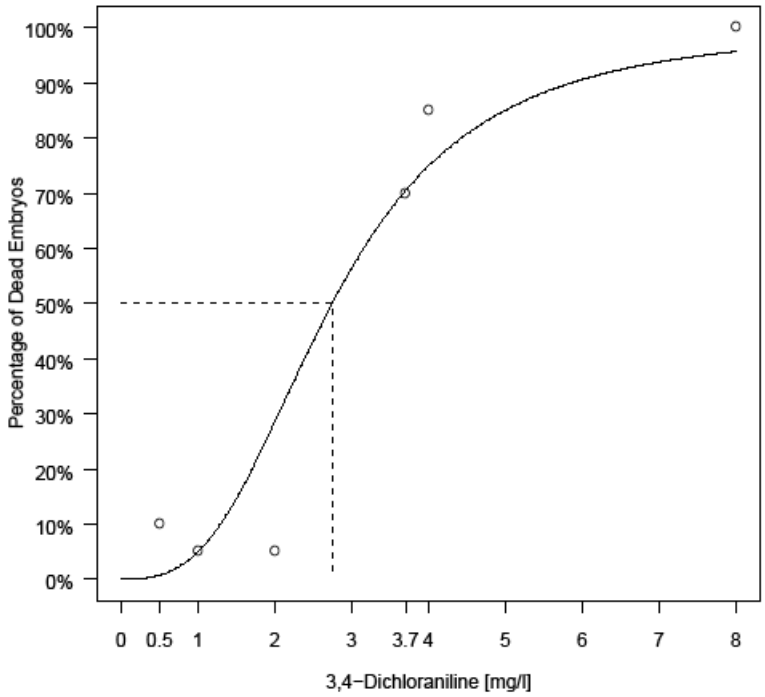


Figure 6b: Laboratory F - single run with 3,4-Dichloroaniline – 96h

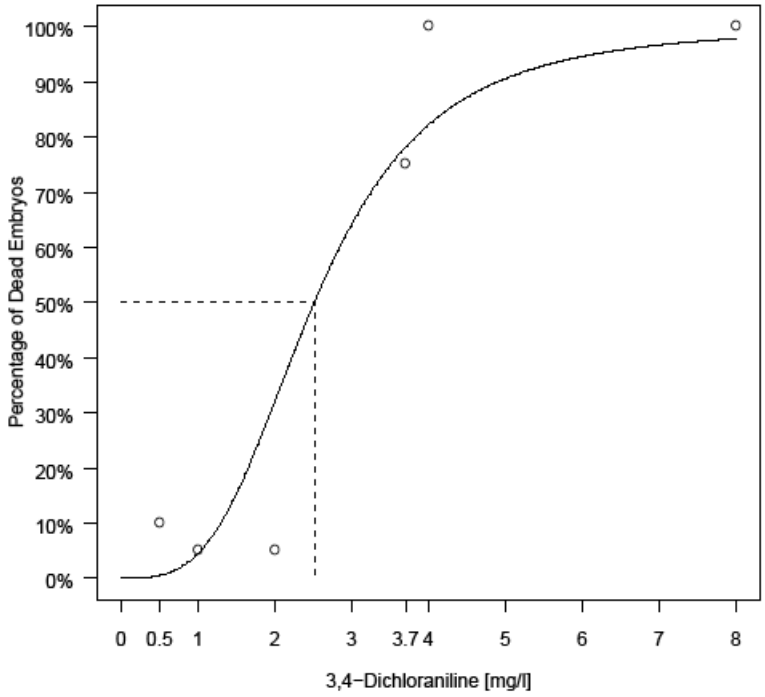


Figure 7a: Laboratory G - single run with 3,4-Dichloroaniline – 48h

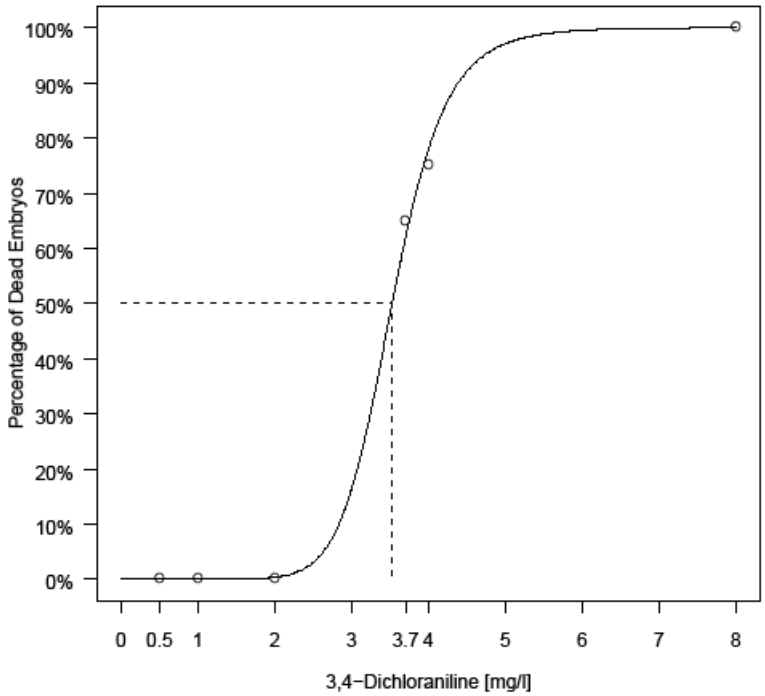


Figure 7b: Laboratory G - single run with 3,4-Dichloroaniline – 96h

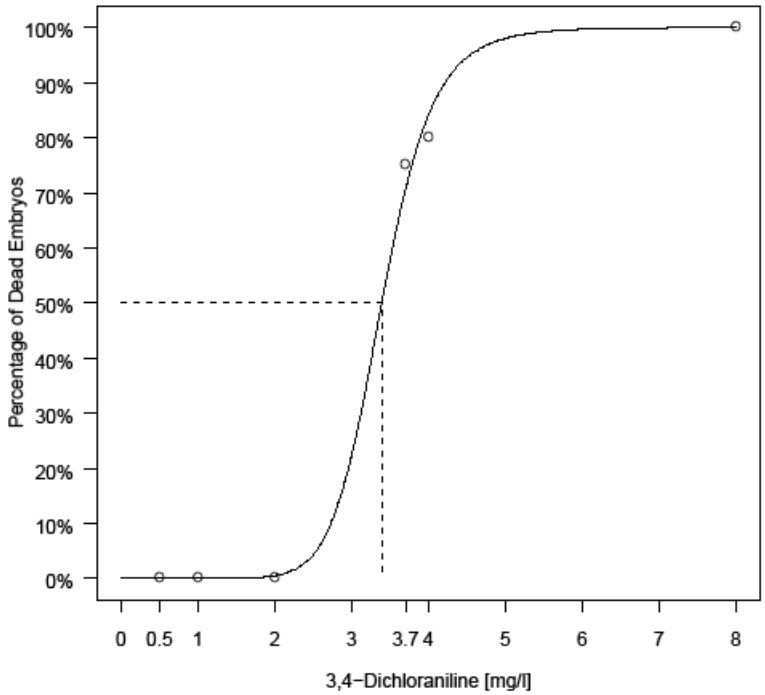


Figure 8a: All laboratories - single run with 3,4-Dichloroaniline – 48h

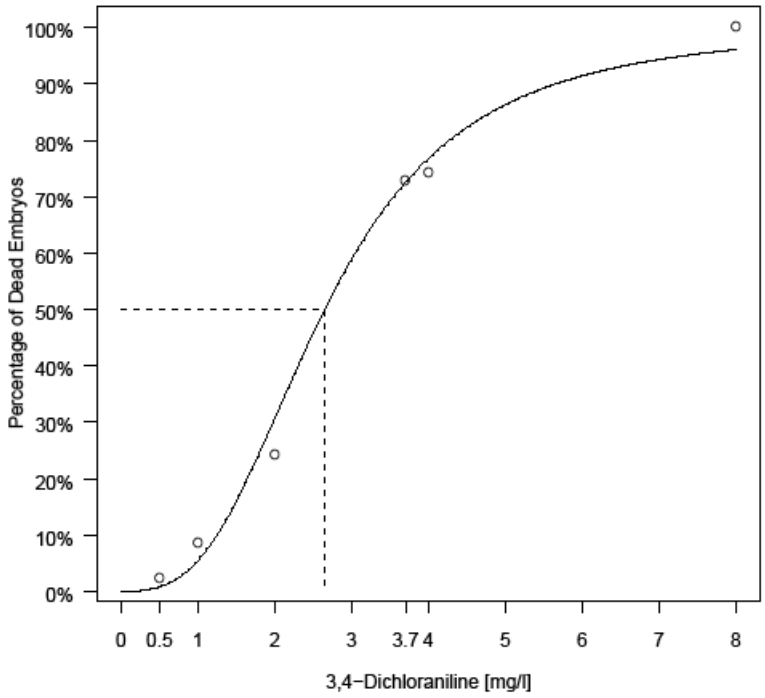
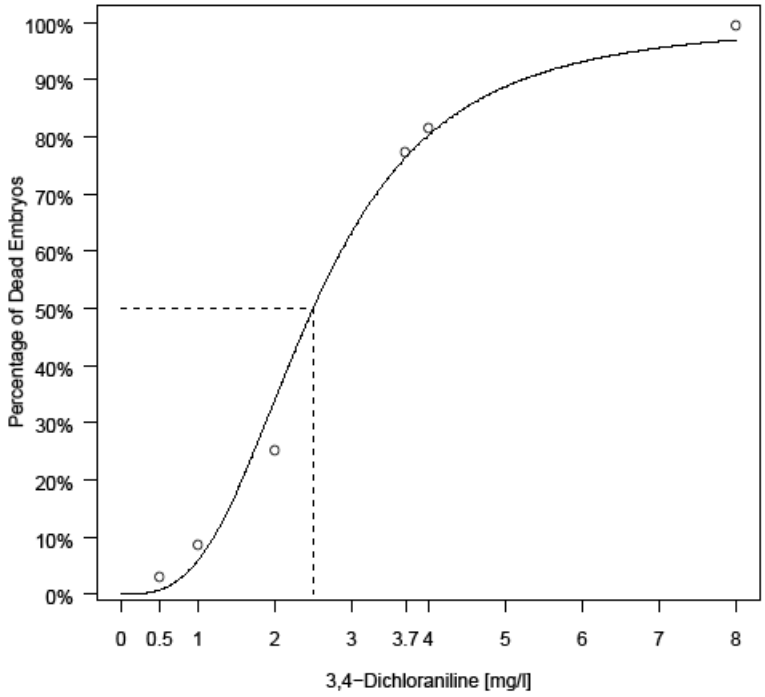


Figure 8b: All laboratories - single run with 3,4-Dichloroaniline – 96h



ANNEX IV - STATISTICAL REPORT PHASE 1A: THREE RUNS WITH 3,4-DCA

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1. Overview

This report refers to the statistical analysis as described in Annex 2 of the trial plan (TP_ZFET_OECD_1a_V01.7). Note that not all calculations were performed for Phase 1a.

2. Inferential statistics

2.1. Choose appropriate model for estimating the LC50 including confidence intervals

The two-parameter log-logistic regression model (Hill-model with the lower limit at 0 and the upper limit at 1) showed in general an appropriate and robust fit whereas the three-parameter log-logistic model (with the upper limit at 1) showed several times a non acceptable fit. Under the assumption that the background mortality is about 2% in the current study, the bias that will be introduced because of leaving out the background mortality parameter in the log-logistic regression model is negligible and has the advantage of a more robust model in general.

Therefore, LC50 values were calculated by the two-parameter log-logistic regression as primary model (*).

(*)Two-parameter log-logistic function with its two parameters: LC50 and the slope (*b*):

$$y = \frac{1}{1+\exp\left[b\left(\log(x)-\log(\mathrm{LC50})\right)\right]}$$

The log-logistic regression model is one of the recommended models by the OECD Series on Testing and Assessment No. 54 for modelling quantal dose-response data [1]. The background mortality (negative controls) was not taken into account, as it does not provide any information for the two-parameter log-logistic regression model [1].

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[1].OECD Series on Testing and Assessment No. 54: Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to application. Chapter 6.2, p 63ff

2.2. Quality criteria for fitting the model

The model fit was visually checked for all the runs using the two-parameter log-logistic regression. If the model shows an obvious inappropriate fit, the estimated LC50 values will be biased. In this situation a three-parameter log-logistic model (with the upper limit at 1) will be used as a secondary model (**).This was performed once for laboratory C, run 1.

(**)Three-parameter log-logistic function with its three parameters: LC50, slope (*b*) and the background response (*c*):

$$y = c\frac{1-c}{1+\exp\left[b\left(\log(x)-\log(\mathrm{LC50})\right)\right]}$$

Convergence of maximization process was checked.

Confidence intervals were calculated using the delta method and the t-distribution as described elsewhere [2-4]. It is well known that in the case of

1. less than two partial effects, and/or
2. one concentration results in zero percent effect and the next higher concentration causes 100% effect

the log-logistic regression can result in too conservative (wide) confidence intervals [5].
It was considered to use other proposed approaches in the two cases described above like the Spearman-Kärber or the binomial method. However, this would result in different point estimates for the LC50 values for the within/between laboratories comparison (reliability) and therefore it was not accomplished.

The calculations were performed with R 2.9.2 and the package drc_1.7-7 with the functions drm() and ED().

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[2] van der Vaart, A. W. (1998): Asymptotic Statistics. Cambridge University Press, Cambridge. Chapter 3
[3] Weisberg, S. (2005): Applied Linear Regression. John Wiley and Sons, New York, third edition. pp. 120-122
[4] Ritz C, Streibing JC. (2008): Nonlinear Regression with R. Springer, New York. Chapter 7.4
[5] Environment Canada (2005 with amendments from 2007): Guidance document on statistical methods for environmental toxicity tests/Method Development and Application Section. Section 4

2.3. Estimate LC50 and confidence intervals per run

The estimated LC50 values and confidence intervals are given in Table 1 for:

- each qualified run at 48 and 96h
- the combined runs per laboratory at 48 and 96h using one two-parameter log-logistic regression model consisting of the qualified results per laboratory
- the overall qualified runs (19) at 48 and 96h using one two-parameter log-logistic regression model consisting of all qualified results

A graphic representation of the results described in Table 1 is given in Figure 1. Individual and combined concentration-response curves are given in Appendix A at the end of this statistical report. The calculated confidence intervals are only of limited value (not very robust). Apparently too wide confidence intervals including possible reasons are noticed in the corresponding tables.

Table 2: LC50 values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with 3,4-DCA

		LC50 3,4-Dichloroaniline [mg/l]				
Laboratory	**Run**	**48h**	**95%CI+-**	**96h**	**95%CI+-**	**Comments**
A	1	2.2	1.2	2.1	1.0	**1**
	2	not qualified		not qualified		
	3	5.4	0.8	5.1	0.7	
combined A	1,3	3.7	0.3	3.5	0.4	
B	1	1.0	0.2	1.1	0.2	
	2	not qualified		not qualified		
	3	1.5	0.3	1.4	0.2	
combined B	1,3	1.2	0.2	1.2	0.1	
C	1	3.7	0.2	2.5	0.4	**2**
	2	3.8	0.3	3.3	0.5	
	3	3.3	0.4	2.5	0.3	
combined C	1,2,3	3.1	0.3	2.4	0.2	
D	1	3.1	0.4	2.6	0.3	
	2	2.5	0.4	2.4	0.3	
	3	2.7	11.1	2.7	10.6	**3**
combined D	1,2,3	2.8	0.2	2.6	0.2	
E	1	5.4	0.8	4.8	0.7	
	2	4.5	0.7	4.1	0.6	
	3	3.6	0.7	3.3	0.6	
combined E	1,2,3	4.5	0.4	4.1	0.4	
F	1	2.5	0.4	2.3	0.3	
	2	2.8	0.5	2.4	0.4	
	3	3.9	0.5	3.2	0.4	
combined F	1,2,3	3.0	0.3	2.6	0.2	
G	1	3.3	0.4	2.8	0.4	
	2	4.5	1.0	3.9	0.2	
	3	4.1	0.3	3.6	0.3	
combined G	1,2,3	4.3	0.3	3.4	0.2	
Overall	19 runs	3.2	0.1	2.7	0.1	

CI: Confidence interval

Comment 1:
For laboratory A run 1, the data showed at 48 and 96h only one partial response. Because of the characteristics of these results the estimated confidence interval is maybe too conservative (wide).

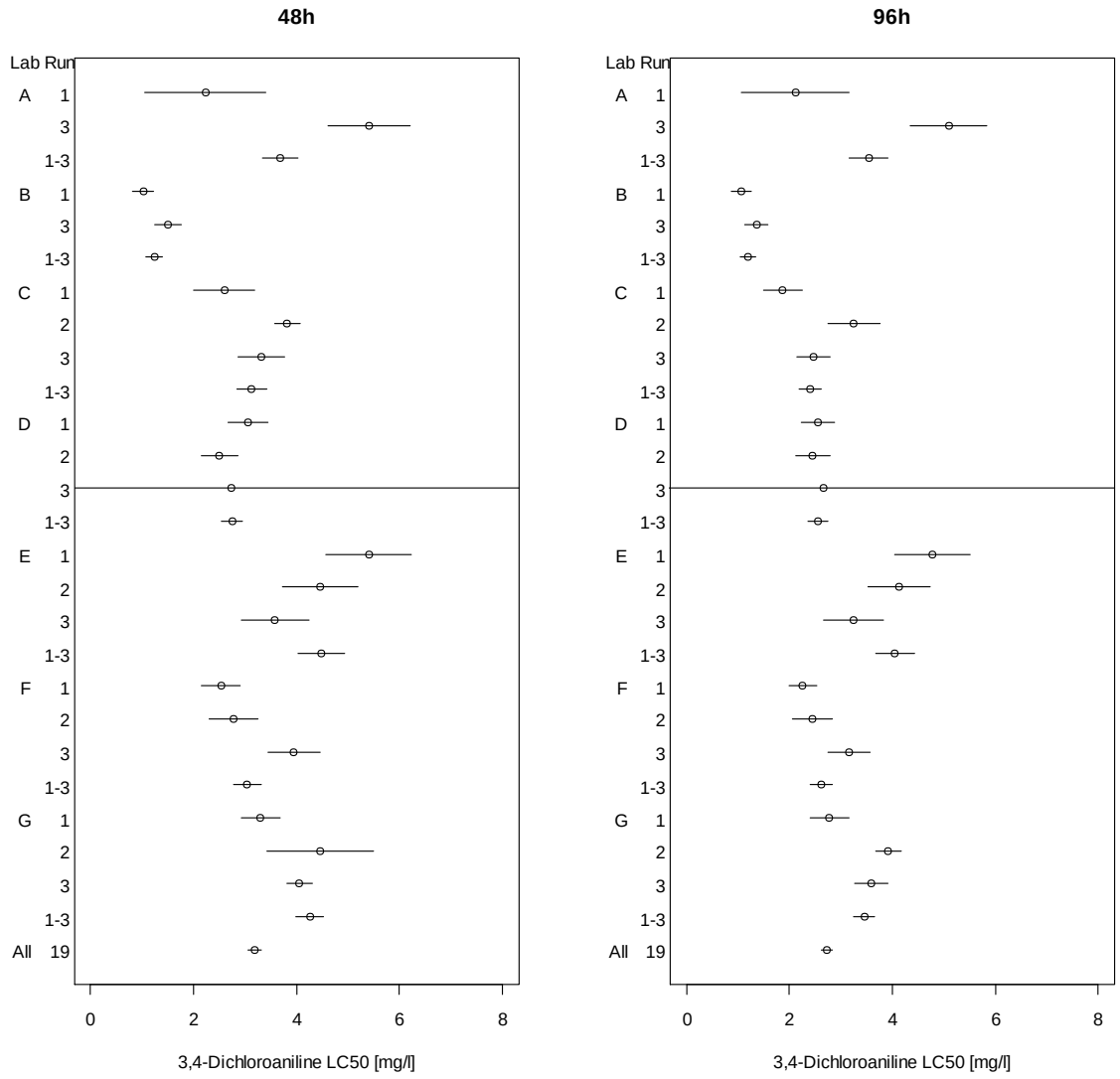
Comment 2:
For laboratory C run 1, the concentration-response curve showed a non acceptable fit (original results: LC50 at 48h: 2.60 mg/l; LC50 at 96h: 1.87 mg/l; data not shown) using the two-parameter log-logistic regression. Therefore, it was replaced by the three-parameter model (see section 2.2.2). For the combined runs (1, 2 and 3), the two-parameter log-logistic regression was used to calculate the concentration-response curve.

Comment 3:
For laboratory D run 3, the data showed at 48 and 96h:

- 0% response at the concentrations 0.5, 1 and 2 mg/l
- 100% response at the concentrations 3.7, 4 and 8 mg/l.

Because of the characteristics of these results the estimated parameters (LC50 and slope) were not significant and this resulted in a very broad confidence interval.

Figure 1: LC50 values and confidence intervals of 3,4-DCA for the Zebrafish Embryo Toxicity Test, Phase 1a, 3 runs



3. Intralaboratory variability

The calculated coefficients of variation per laboratory are given in Table 2.

Table 2: Intralaboratory reproducibility - coefficients of variation for 3,4-DCA – three runs

	Intralaboratory Coefficient of Variation	
	48h	**96h**
Laboratory A	58.8%	58.5%
Laboratory B	27.2%	17.1%
Laboratory C	7.3%	16.6%
Laboratory D	10.0%	4.4%
Laboratory E	20.4%	18.9%
Laboratory F	24.6%	17.9%
Laboratory G	14.9%	17.2%

Laboratory A & B: only two qualified runs

4. Interlaboratory variability

The interlaboratory coefficients of variation were calculated based on the combined LC50 calculations (see Table 1) and are given in Table 3.

Table 3: Interlaboratory reproducibility - coefficients of variation for 3,4-DCA – three runs

	Interlaboratory Coefficient of Variation	
	48h	***96h***
All laboratories	*33.7%*	*33.4%*
Only laboratories with three qualified runs (C-G)	*22.1%*	*23.6%*

An ANOVA was calculated based upon the results of laboratories C, D, E, F and G (48h: $p = 0.044$, 96h $p = 0.023$).

Bonferroni's multiple comparison tests between all pairs of laboratories showed at 48 and 96h no significant pairs.

5. Estimation of possible concentration and acceptance criteria for 3,4-DCA at 96h as positive control for the next phases of the study

NOTE: The following proposals should serve as a basis for discussion and will need some feedback from the VMG.

Step 1: Test concentration at 96h

Proposal:

Use of one of the tested 3,4-DCA concentrations as the reference test concentration for the positive control: This should be preferred over non-tested concentrations since empirical test results are available. Therefore no interpolation would be necessary.

It is advised that the positive control concentration should be within >50% and <100% of lethality. The 4.0 mg/l test concentration of 3,4-DCA at 96h resulted in a 82.2% mortality rate for the qualified runs (309 out of 376 or 16.4 dead embryos out of 20 exposed embryos; see Table 4).

Table 4: Lethal effects of 3.4-DCA at 4.0 mg/l at 96h on the fish embryos as reported by the participating laboratories

3,4-DCA concentration (mg/l)	Lethal effects	Max	Laboratory	Run	Time (h)
4.0	17	17	a	1	96
4.0	Not qualified		a	2	96
4.0	3	20	a	3	96
4.0	20	20	b	1	96
4.0	Not qualified		b	2	96
4.0	20	20	b	3	96
4.0	20	20	c	1	96
4.0	17	19	c	2	96
4.0	19	20	c	3	96
4.0	20	20	d	1	96
4.0	20	20	d	2	96
4.0	20	20	d	3	96
4.0	7	20	e	1	96
4.0	10	20	e	2	96
4.0	14	20	e	3	96
4.0	20	20	f	1	96
4.0	19	20	f	2	96
4.0	16	20	f	3	96
4.0	20	20	g	1	96
4.0	11	20	g	2	96
4.0	16	20	g	3	96
Total	309	376			

Using the two-parameter log-logistic regression model including all qualified data for all concentrations from all laboratories, a tested concentration of 4.0 mg/l would correspond to a lethality of 79.6% for the exposed embryos at 96h (see Figure 1 in Appendix A).

The empirical distribution at 4.0 mg/l (82.2%) and the model/based calculations (LC79.6. = 4.0 mg/l) showed comparable results.

Step 2: Positive acceptance criterion at 96h

Based on the statistical assumption that one or two of the 19 runs would be non-acceptable regarding the positive acceptance criteria, this would give a frequency of roughly 5 or 10% rejected runs.
Based on standardized results to 20 embryos, the 5% quantiles calculated from the empirical distribution at a test concentration of 4.0 mg/l is 6.6 dead embryos out of 20 exposed embryos (33%). Other calculated quantiles from the empirical distribution are given in Table 5.

Table 5: Empirical quantiles calculated from the empirical results at a test concentration 4.0 mg/l at 96h standardized on 20 embryos

Empirical quantiles	1%	2.5%	5%	10%
x of 20 embryos	3.7	4.8	6.6	9.4

Two situations are considered:

An acceptance criterion of at least 6 dead embryos out of 20 exposed embryos (30%) or 7 dead embryos out of 20 exposed embryos (35%) would result in one non-qualified run (Laboratory A, run 3: 3 dead embryos out of 20 exposed embryos- see Table 4).

An acceptance criterion of at least 8 dead embryos out of 20 exposed embryos (40%) would result in two non-qualified runs (11.1%: Laboratory A, run 3 and Laboratory E, run 1: 7 dead embryos out of 20 exposed embryos- see Table 4). Moreover, these two runs resulted in the highest calculated LC50 concentrations (see Table 1).

Following the idea of a conservative approach to increase the quality and reliability of the test results a proposal could be:

A test concentration of 4.0 mg/l 3,4-DCA for the positive control should result in 80% mortality rate at 96h. The assay is acceptable if the positive control shows at least a mortality rate at 96h of 8 dead embryos out of 20 exposed embryos (40%).

NOTE: Confidence and tolerance intervals will be calculated for the final report

6. Alternate estimation of possible concentration and acceptance criteria for 3,4-DCA at 48h as positive control

NOTE: The following proposals should serve as a basis for discussion and will need some feedback from the VMG.

Step 1: Test concentration at 48h

Proposal:

Use of one of the tested 3,4-DCA concentrations as the reference test concentration for the positive control: This should be preferred over non-tested concentrations since empirical test results are available. Therefore no interpolation would be necessary.

It is advised that the positive control concentration should be within >50% and <100% of lethality. The 4.0 mg/l test concentration of 3,4-DCA at 48h resulted in a 67.6% mortality rate for the qualified runs (256 out of 379 or 13.5 dead embryos out of 20 exposed embryos; see Table 4).

Table 6: Lethal effects of 3.4-DCA at 4.0 mg/l at 48h on the fish embryos as reported by the participating laboratories

3,4-DCA concentration (mg/l)	Lethal effects	Max	Laboratory	Run	Time (h)
4.0	20	20	a	1	48
4.0	Not qualified		a	2	48
4.0	1	20	a	3	48
4.0	20	20	b	1	48
4.0	Not qualified		b	2	48
4.0	20	20	b	3	48
4.0	16	20	c	1	48
4.0	11	19	c	2	48
4.0	17	20	c	3	48
4.0	18	20	d	1	48
4.0	20	20	d	2	48
4.0	20	20	d	3	48
4.0	3	20	e	1	48
4.0	8	20	e	2	48
4.0	13	20	e	3	48
4.0	17	20	f	1	48
4.0	16	20	f	2	48
4.0	11	20	f	3	48
4.0	18	20	g	1	48
4.0	3	20	g	2	48
4.0	4	20	g	3	48
Total	256	379			

Using the two-parameter log-logistic regression model including all qualified data for all concentrations from all laboratories, a tested concentration of 4.0 mg/l would correspond to a lethality of 66.8% for the exposed embryos at 48h (see Figure 1 in Appendix A).

The empirical distribution at 4.0 mg/l (67.6%) and the model/based calculations (LC66.8 = 4.0 mg/l) showed comparable results.

Step 2: Positive acceptance criterion at 48h

Based on the statistical assumption that one or two of the 19 runs would be non-acceptable regarding the positive acceptance criteria, this would give a frequency of roughly 5 or 10% rejected runs.
Based on standardized results to 20 embryos, the 5% quantiles calculated from the empirical distribution at a test concentration of 4.0 mg/l is 2.8 dead embryos out of 20 exposed embryos (14%). Other calculated quantiles from the empirical distribution are given in Table 5.

Table 7: Empirical quantiles calculated from the empirical results at a test concentration 4.0 mg/l at 48h standardized on 20 embryos

Empirical quantiles	1%	2.5%	5%	10%
x of 20 embryos	1.4	1.9	2.8	3

An acceptance criterion of at least 3 dead embryos out of 20 exposed embryos (15%) would result in one non-qualified run (Laboratory A, run 3: 1 dead embryos out of 20 exposed embryos- see Table 4).

NOTE: Confidence and tolerance intervals will be calculated for the final report.

7. Test of effect on internal controls caused by the increasing test concentrations

The inferential statistics as described in the Annex 2, chapter 3.4 and 3.5 of the trial plan (TP_ZFET_OECD_1a_V01.7) and are given in Table 6. Summaries of the raw data are given in the tables 7 and 8 respectively.

Table 8: One sided p-values of the statistical tests for cross-contamination

Statistical test	Sample	Time	p-value
Stratified exact Cochran-Armitage trend test	All Laboratories	48h	**0.009**
	Only Laboratories with 3 qualified runs (C-G)		**0.008**
	All Laboratories	96h	**0.009**
	Only Laboratories with 3 qualified runs (C-G)		**0.019**
Exact Fisher test	All Laboratories	48h	0.164
	Only Laboratories with 3 qualified runs (C-G)		**0.045**
	All Laboratories	96h	0.081
	Only Laboratories with 3 qualified runs (C-G)		**0.045**

p-values < 0.05 are indicated by bold font (significant).

The Cochran-Armitage trend test showed a significant concentration-dependent cross-contamination effect on the internal controls.

The Fisher test of internal vs. external controls showed only a weak effect. A homogeneity of Odds-Ratios test have not be performed because for some laboratories the Odds-Ratio cannot be defined or its infinity.

The plate design for the next phases of the study, as for the previous phase, should take into account that cross-contamination effects cannot be excluded.

Table 9: Overview internal and external controls per laboratory

Controls	Time	Laboratory	Embryos Dead	Embryos Survival
External	48h	a	0	48
		b	1	47
		c	4	68
		d	0	72
		e	4	68
		f	1	71
		g	1	71
	96h	a	0	48
		b	3	45
		c	4	68
		d	0	72
		e	4	68
		f	1	71
		g	1	71
Internal	48h	a	0	48
		b	3	45
		c	1	71
		d	0	72
		e	0	72
		f	1	71
		g	1	71
	96h	a	0	48
		b	3	45
		c	1	71
		d	0	72
		e	0	72
		f	1	71
		g	1	71

Table 10: Summarised overview internal and external controls

Sample	Controls	Time	Embryos Dead	Embryos Survival	Fraction
All Laboratories	External	48h	11	445	2.41%
		96h	13	443	2.85%
	Internal	48h	6	450	1.32%
		96h	6	450	1.32%
Only Laboratories with complete datasets (C-G)	External	48h	10	350	2.78%
		96h	10	350	2.78%
	Internal	48h	3	357	0.83%
		96h	3	357	0.83%

Appendix A: Phase 1a – Three Runs with 3,4-DCA
Individual and Combined Concentration-Response Curves

Figure 1: All laboratories - Combined concentration-response curves at 48h and 96h based on 19 qualified runs from 7 laboratories

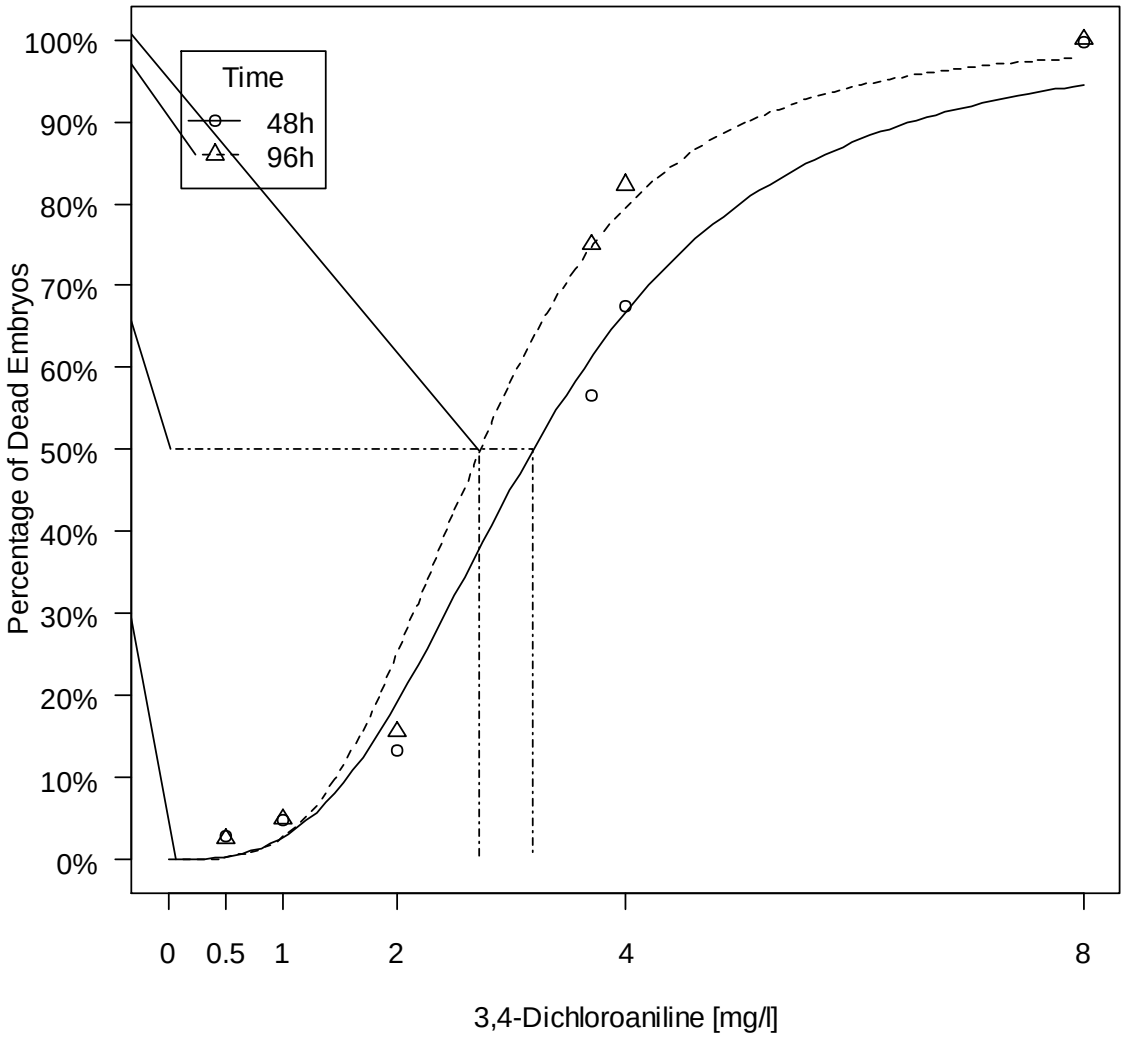


Figure 2a: Laboratory A - Combined concentration-response curves at 48h and 96h based on 2 qualified runs

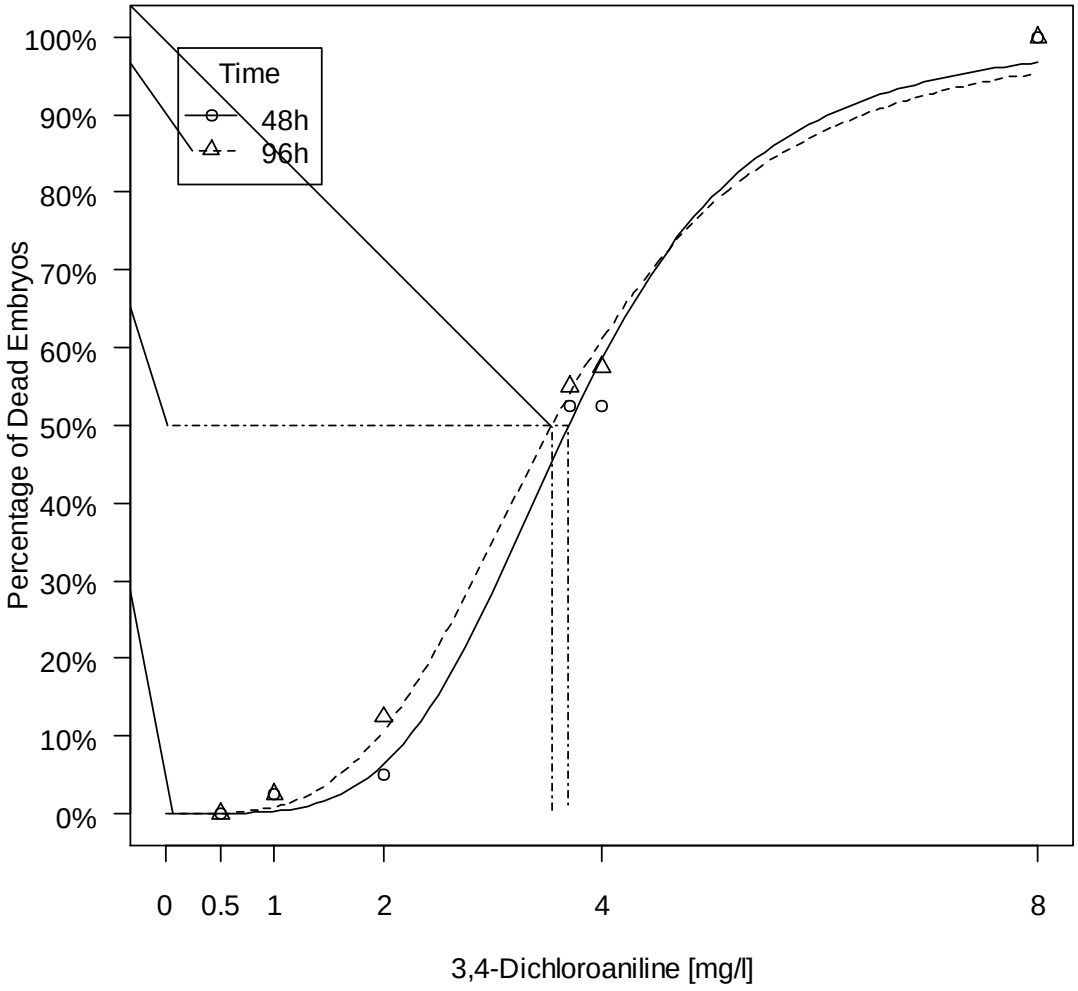


Figure 2b: Laboratory A - Individual concentration-response curves at 48h based on 2 qualified runs

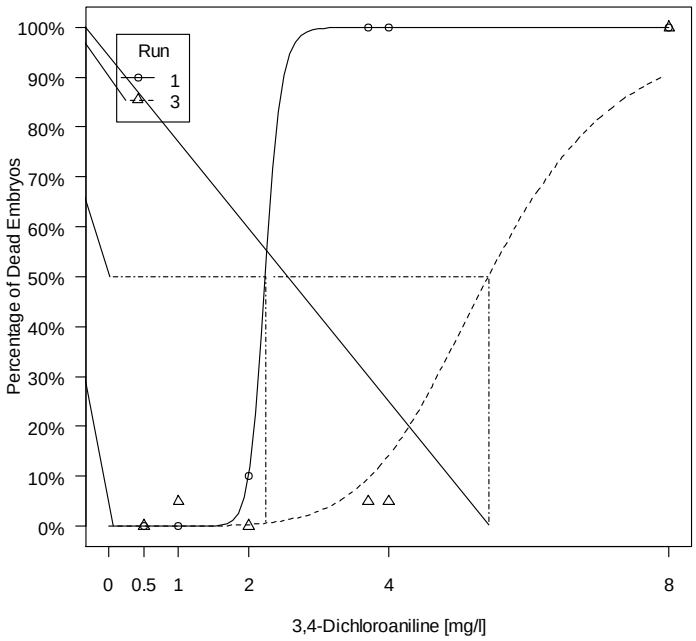


Figure 2c: Laboratory A - Individual concentration-response curves at 96h based on 2 qualified runs

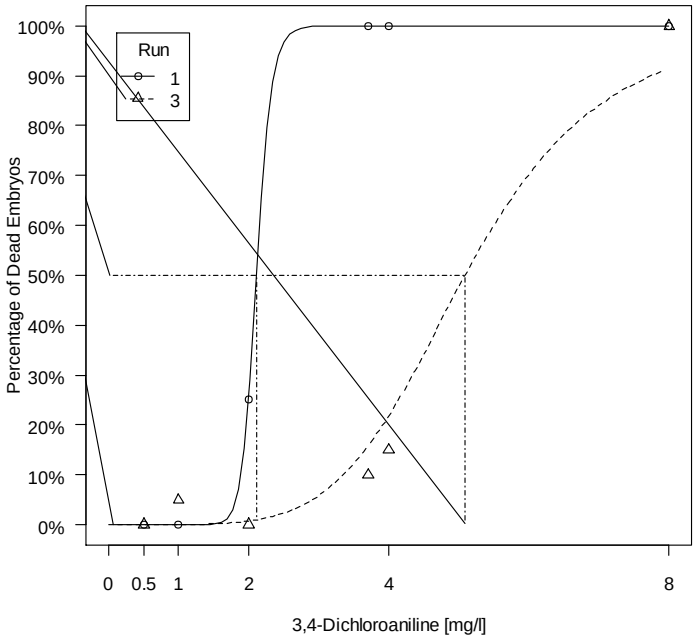


Figure 3a: Laboratory B - Combined concentration-response curves at 48h and 96h based on 2 qualified runs

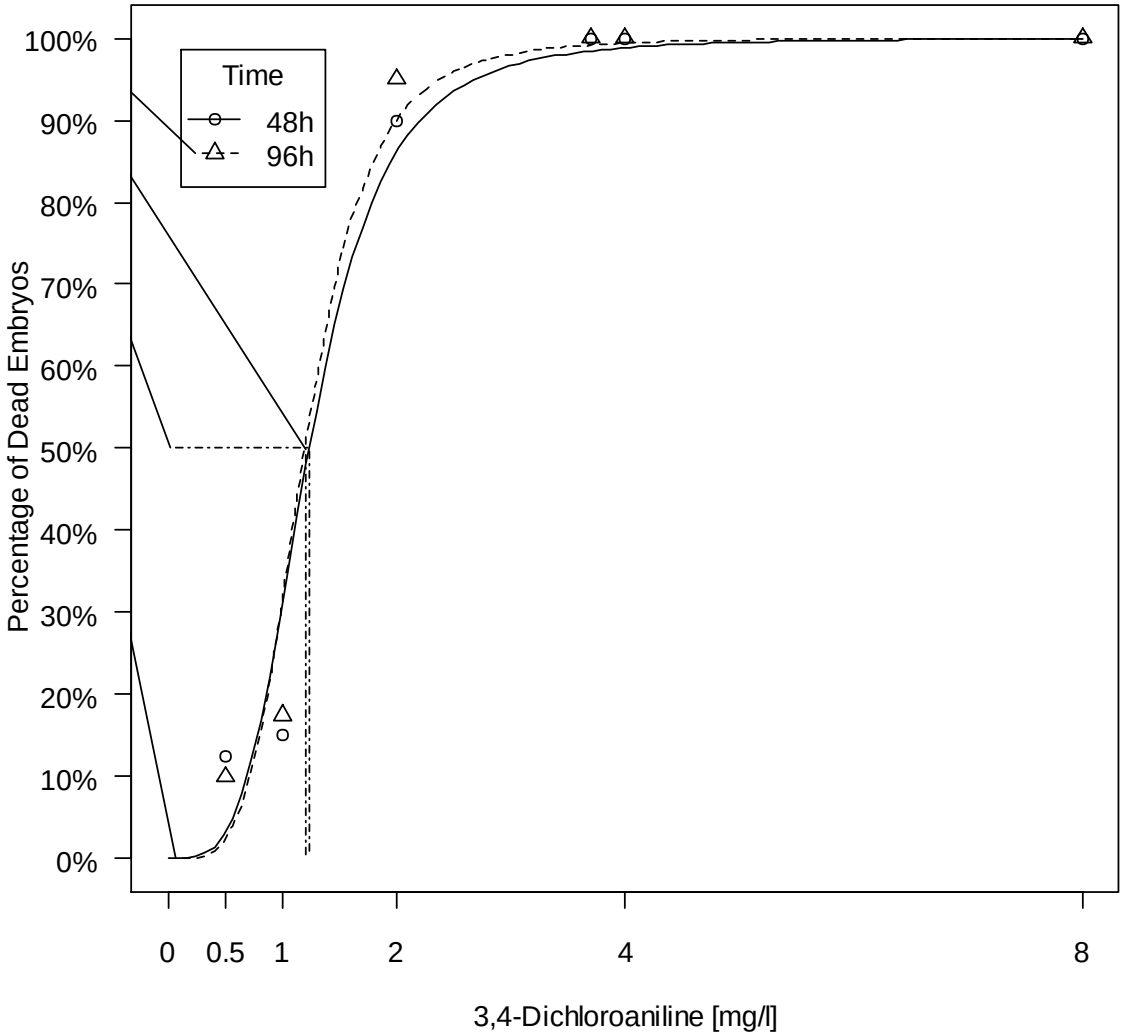


Figure 3b: Laboratory B - Individual concentration-response curve at 48h based on 2 qualified runs

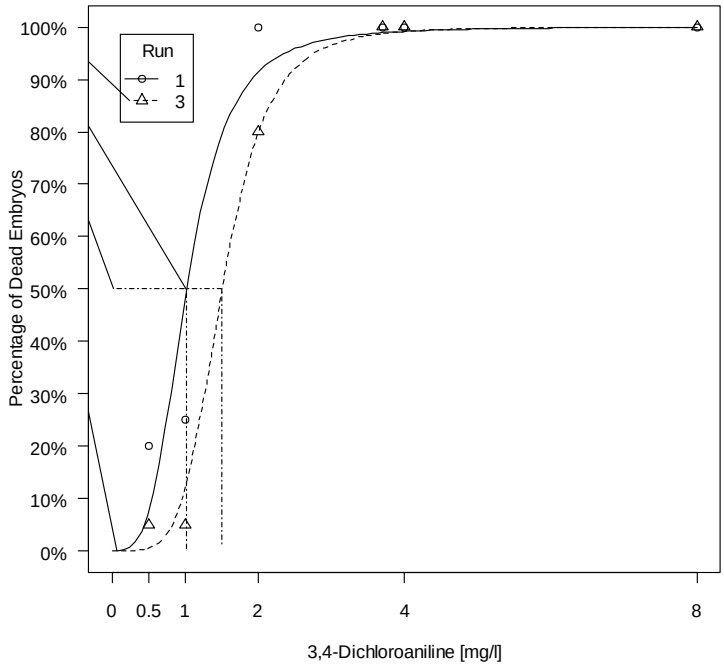


Figure 3c: Laboratory B - Individual concentration-response curve at 96h based on 2 qualified runs

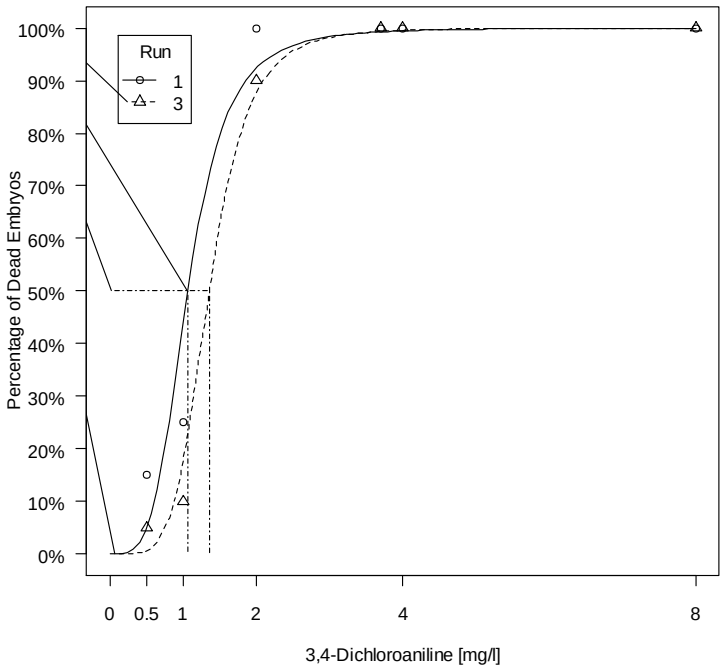


Figure 4a: Laboratory C - Combined concentration-response curves at 48h and 96h based on 3 qualified runs

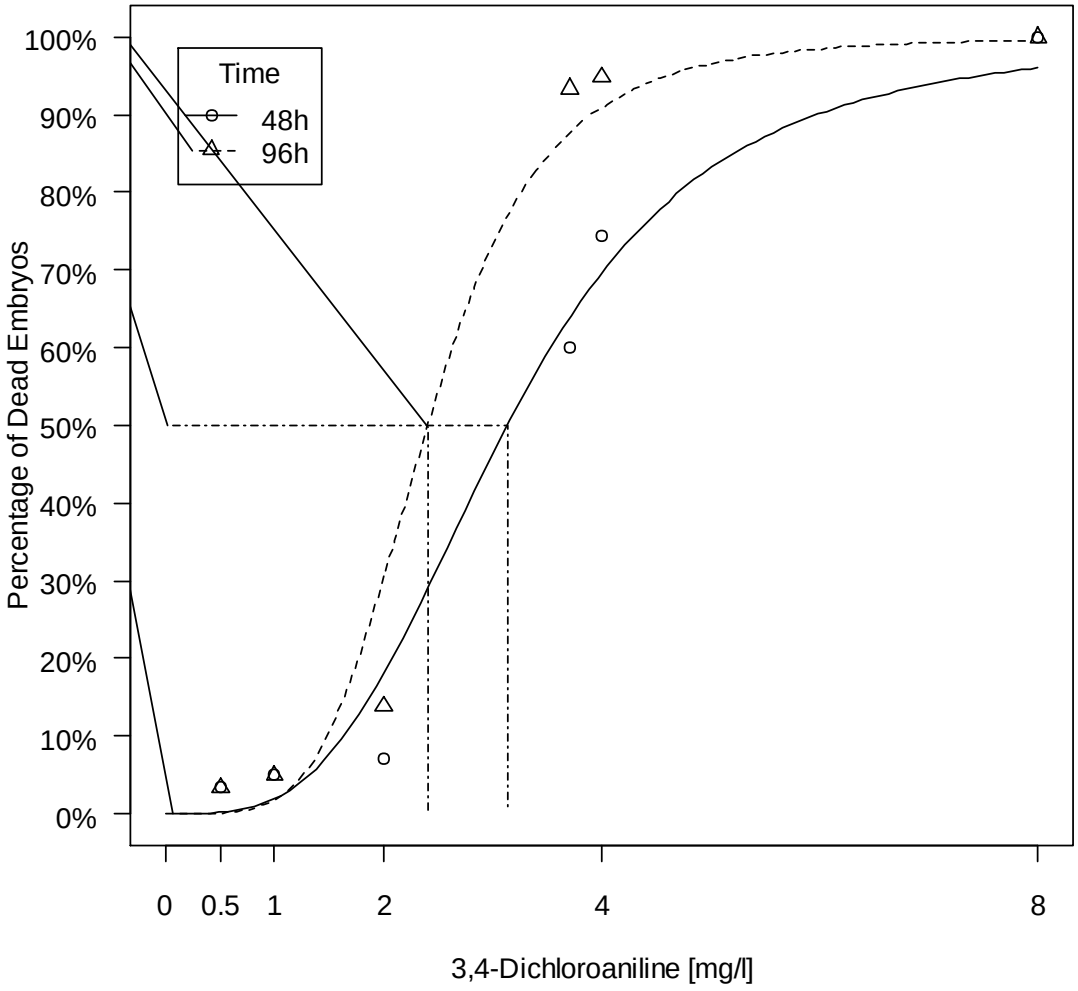


Figure 4b: Laboratory C - Individual concentration-response curves at 48h based on 3 qualified runs

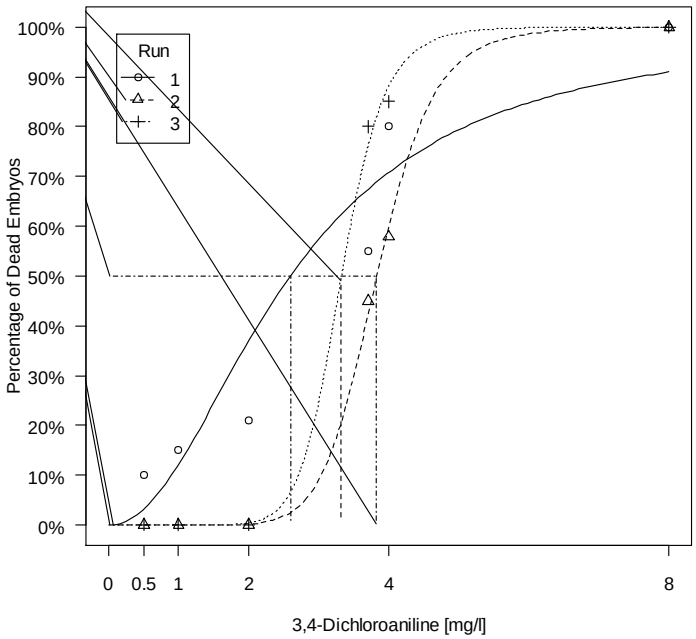


Figure 4c: Laboratory C - Individual concentration-response curves at 96h based on 3 qualified runs

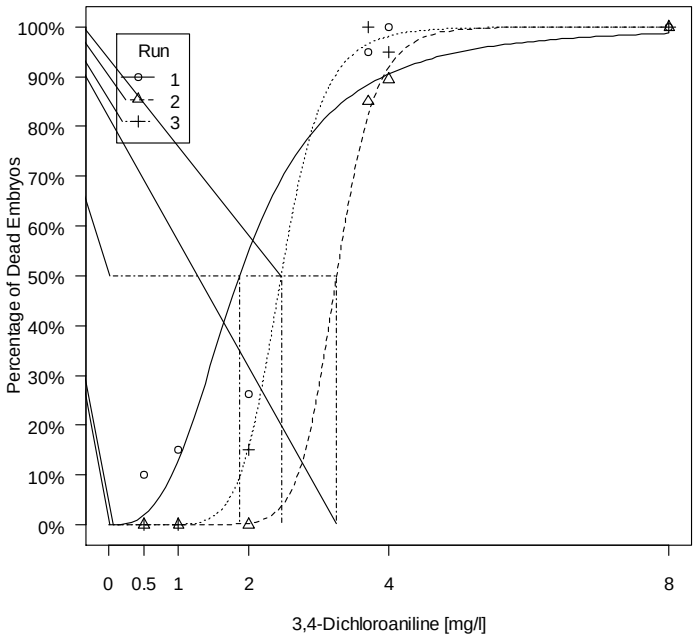
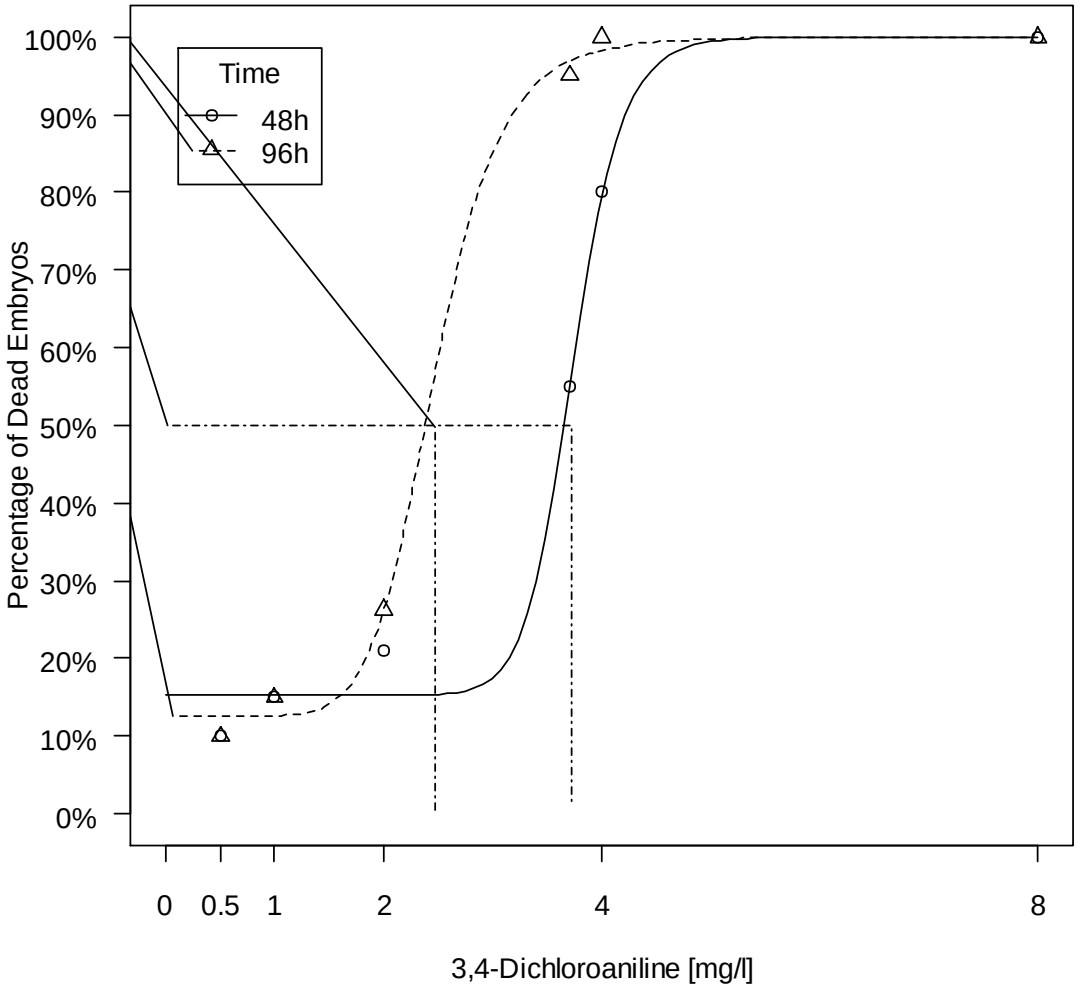


Figure 4d: Laboratory C – Alternative individual concentration-response curves at 48h and 96h based on run n°1



Alternative calculation based on the three-parameter logistic function where the upper limit is equal to 1.

Figure 5a: Laboratory D - Combined concentration-response curves at 48h and 96h based on 3 qualified runs

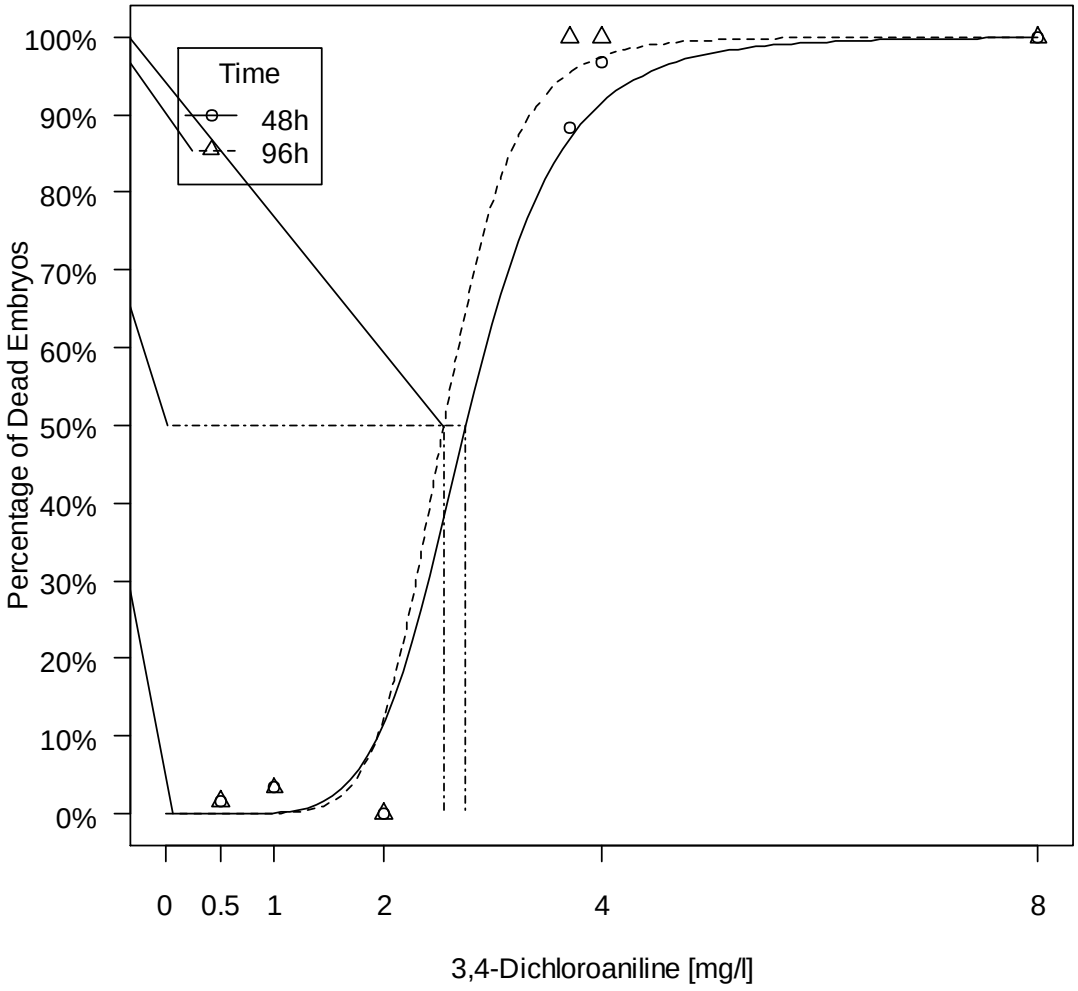


Figure 5b: Laboratory D - Individual concentration-response curves at 48h based on 3 qualified runs

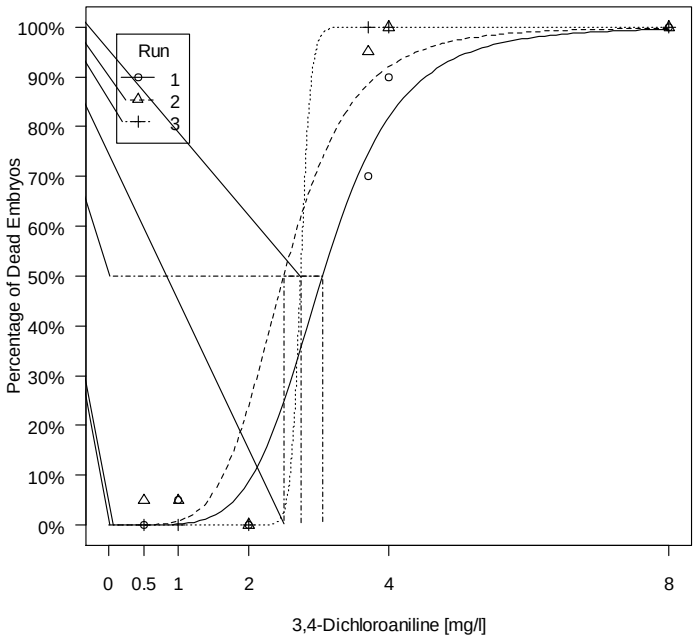


Figure 5c: Laboratory D - Individual concentration-response curves at 96h based on 3 qualified runs

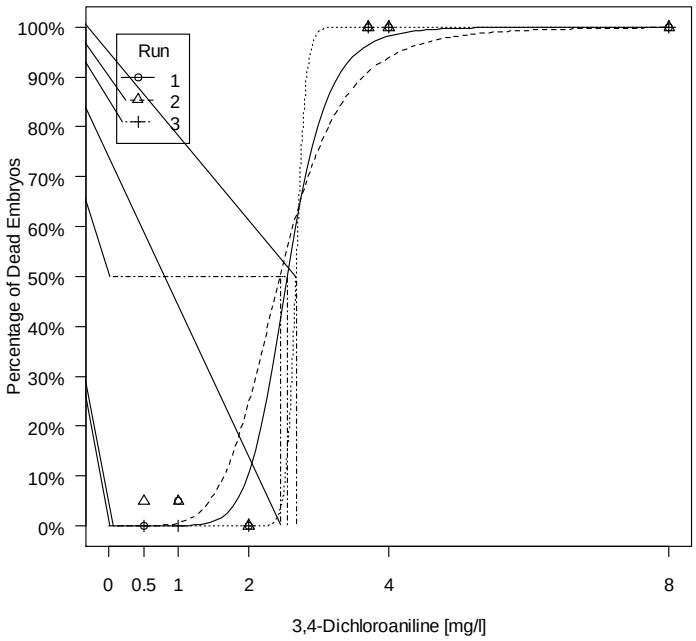


Figure 6a: Laboratory E - Combined concentration-response curves at 48h and 96h based on 3 qualified runs

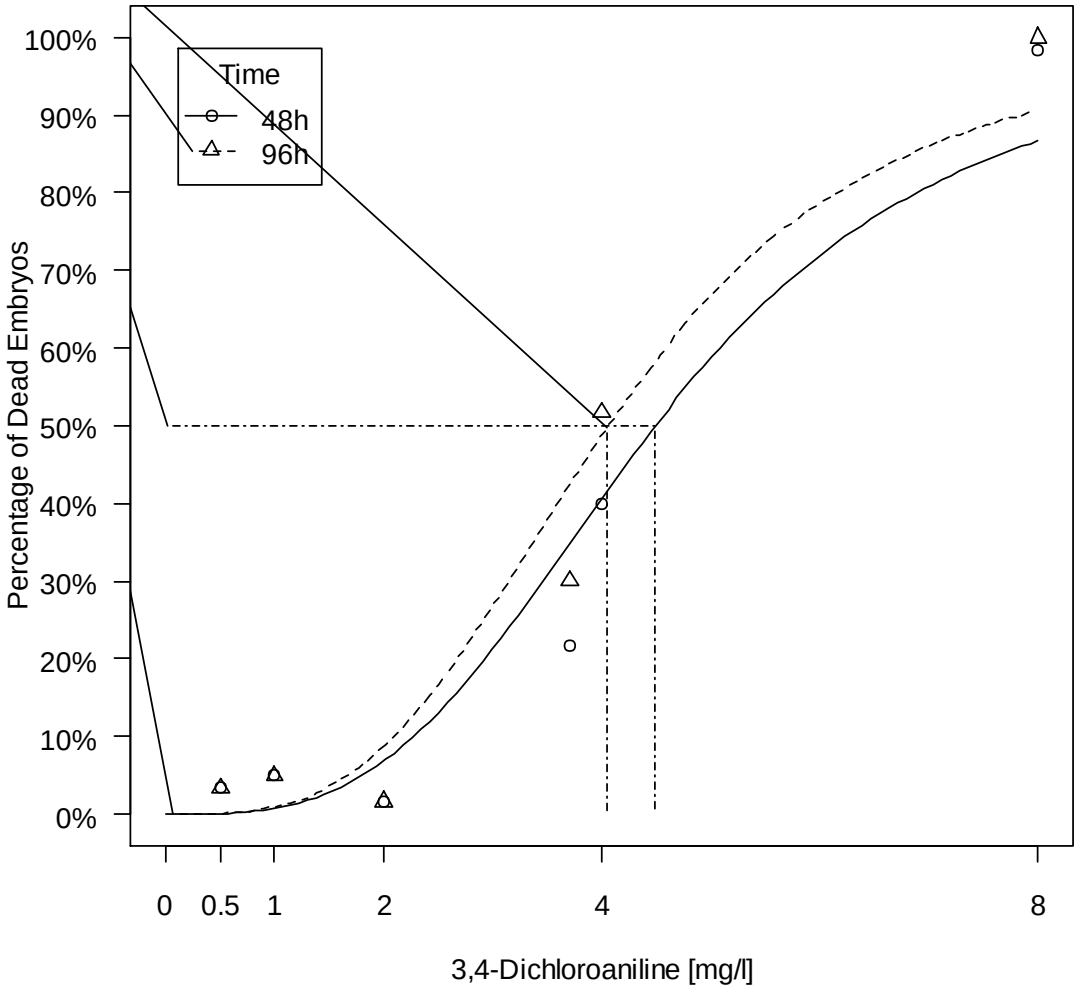


Figure 6b: Laboratory E - Individual concentration-response curves at 48h based on 3 qualified runs

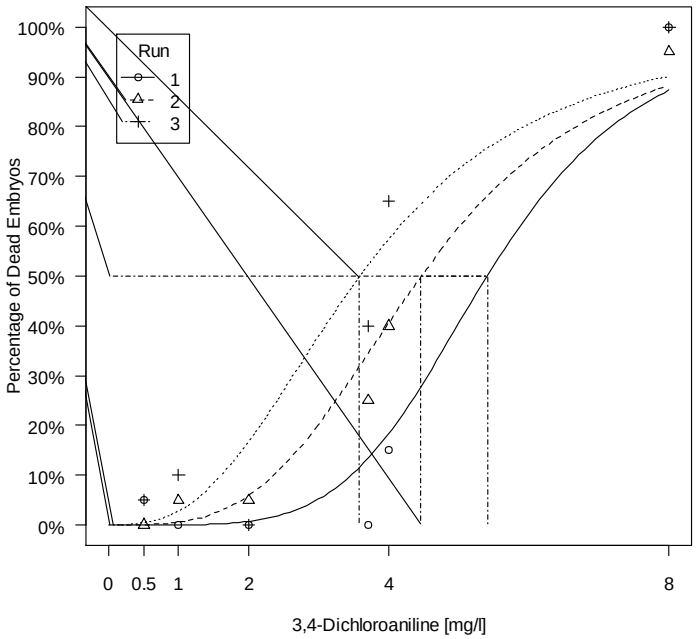


Figure 6c: Laboratory E - Individual concentration-response curves at 96h based on 3 qualified runs

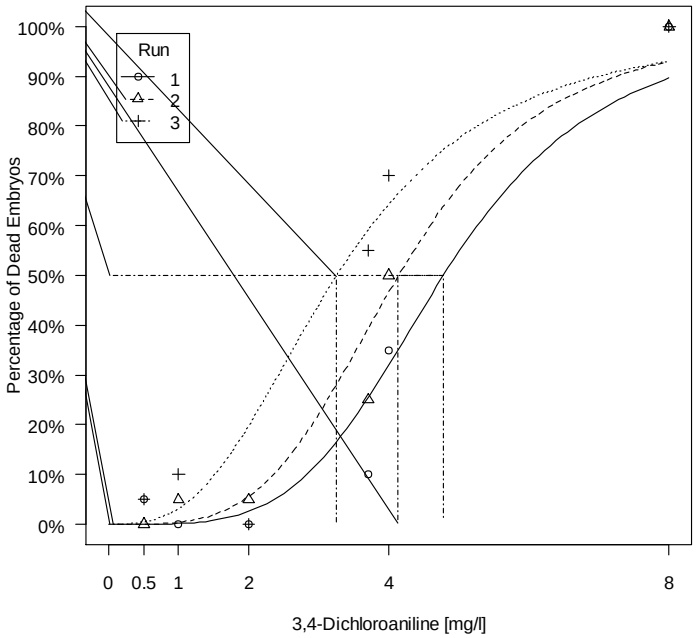


Figure 7a: Laboratory F - Combined concentration-response curves at 48h and 96h based on 3 qualified runs

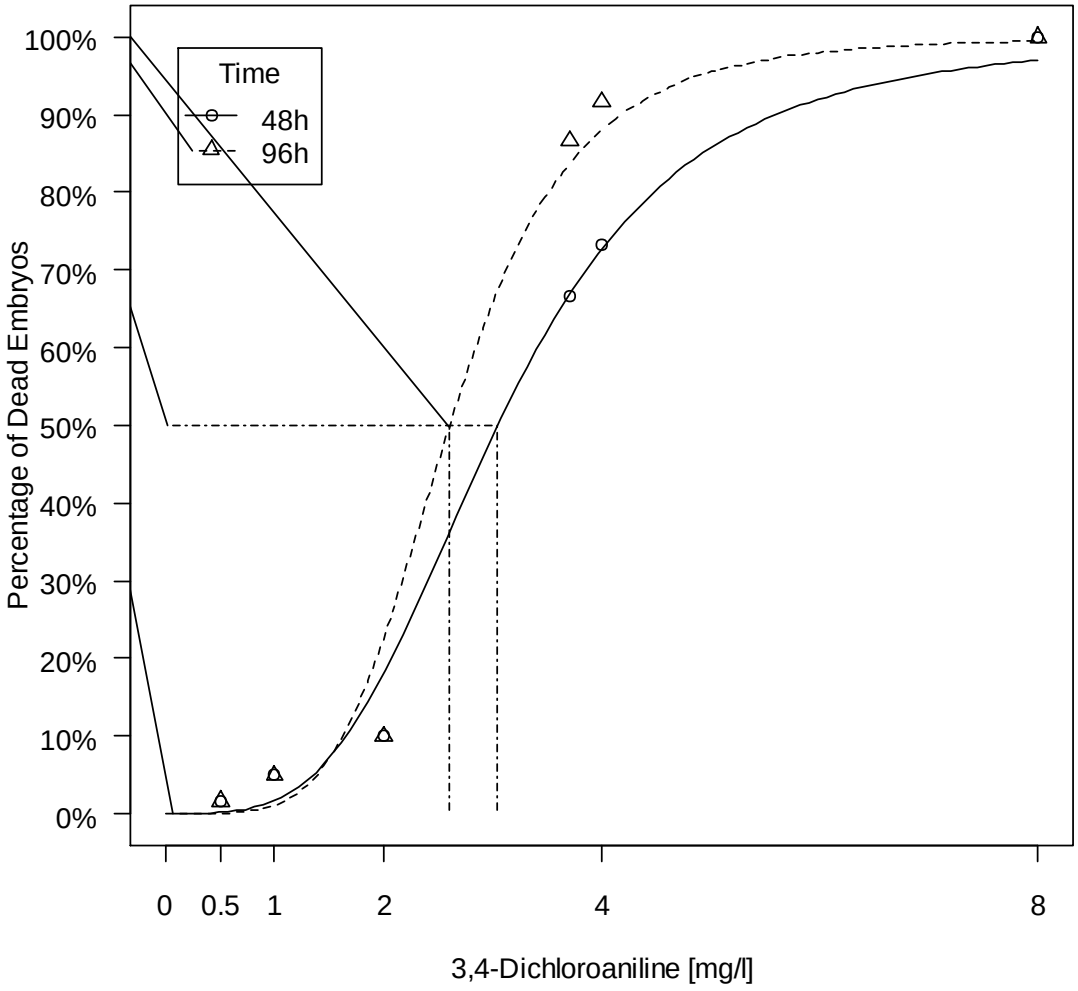


Figure 7b: Laboratory F - Individual concentration-response curves at 48h based on 3 qualified runs

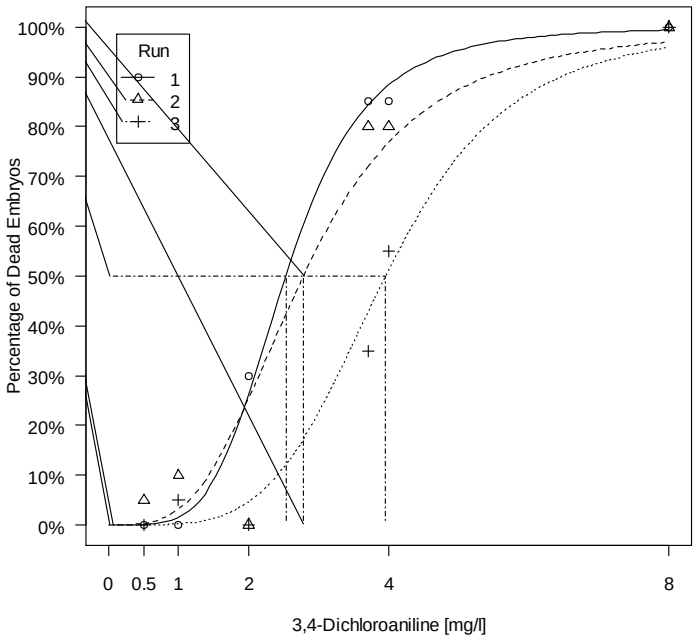


Figure 7c: Laboratory F - Individual concentration-response curves at 96h based on 3 qualified runs

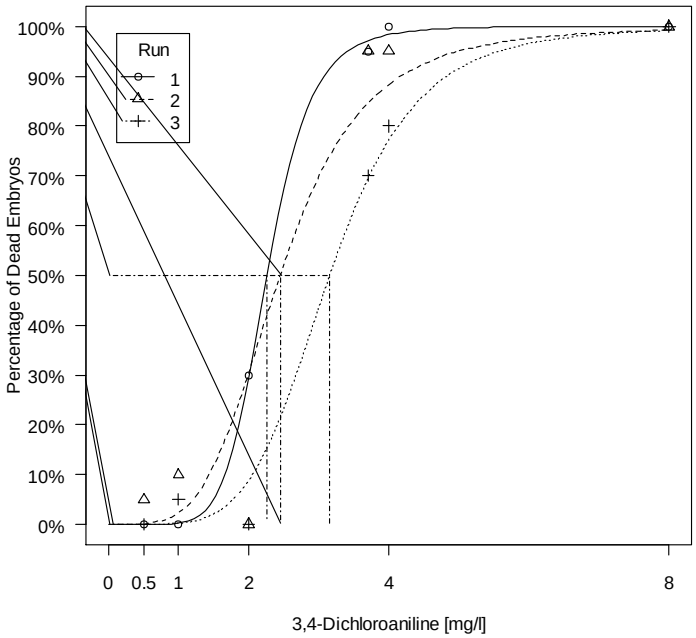


Figure 8a: Laboratory G - Combined concentration-response curves at 48h and 96h based on 3 qualified runs

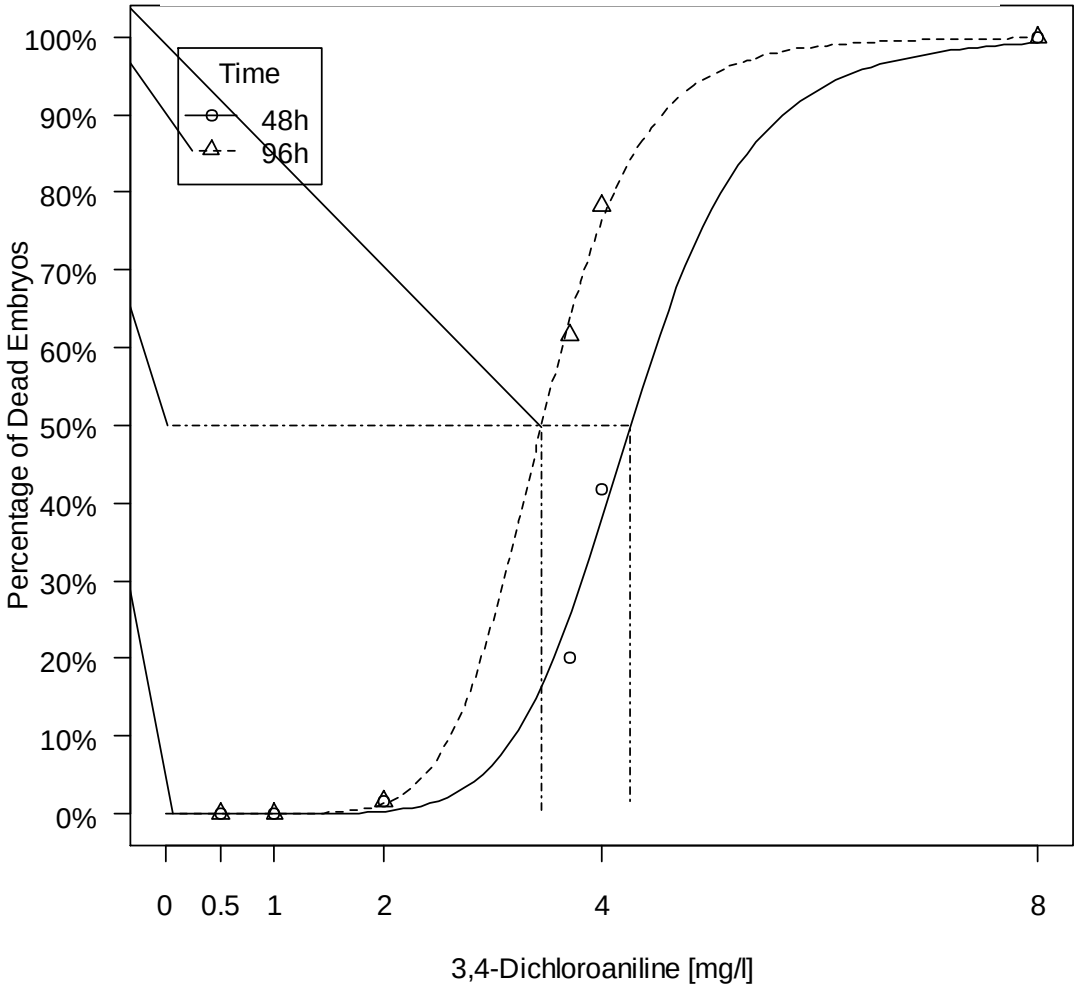


Figure 8b: Laboratory G - Individual concentration-response curves at 48h based on 3 qualified runs

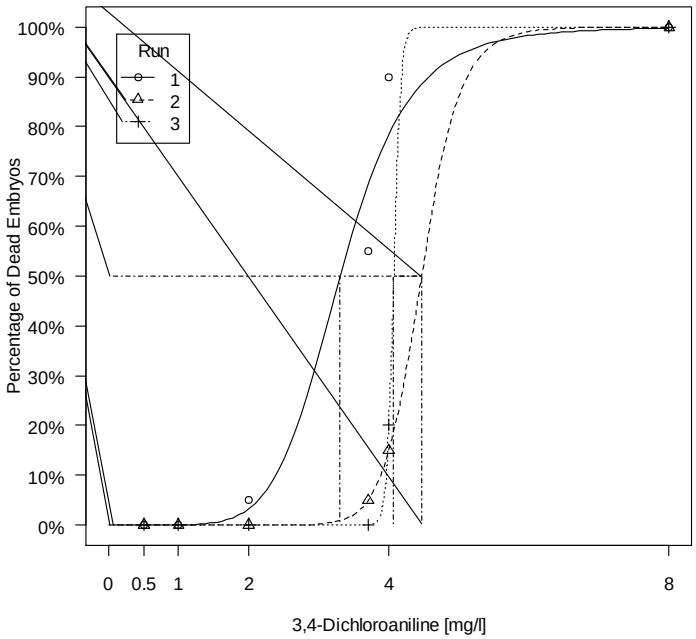
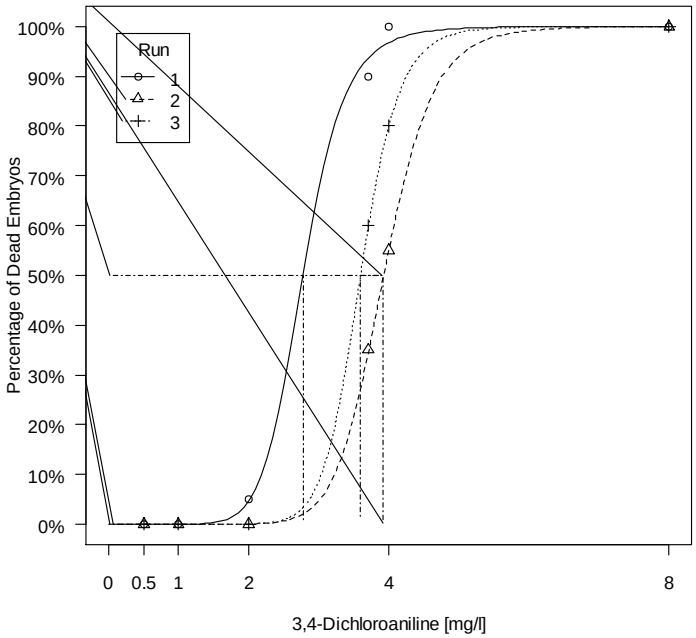


Figure 8c: Laboratory G - Individual concentration-response curves at 96h based on 3 qualified runs



ANNEX V - ANALYSIS OF 6 CHEMICALS IN FET STOCK AND EXPOSURE SOLUTIONS FOR PHASE 1B

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Draft: 27 July 2010

1-INTRODUCTION

Analytical verification of six different compounds in aqueous stock and exposure solutions utilized during an international validation study of the FET was performed at Procter & Gamble. Analyses were conducted using viable non-specific methods in Central Product Safety (Miami Valley Innovation Center, Cincinnati, Ohio) or by the Trace Analytical Core (Mason Business Center, Cincinnati, Ohio USA).
Previously, 3,4-DCA (dichloroaniline), which is used as an internal positive control test chemical in the FET (OECD 2009; *Zebrafish Embryo Toxicity Test*, Standard Operation Procedure, SOP ZFET OECD V02.7, April 28th, 2009 and V02.8, June 17th, 2009), was assessed under similar circumstances in Phase 1a (Transferability Assessment) and summarized in (OECD 2010). These studies confirmed that stock solutions were reliably produced in participating laboratories that successfully shipped samples to the analytical laboratory. Inter-laboratory differences were relatively small and would have little impact on execution of dilution series across laboratories. It is highly likely that confirmation of exposures in the tests themselves would also be similar. Stock solutions of 3,4-DCA declined through time. Measurement of 3,4-DCA concentrations in multi-well plates during FET exposures were determined under static and static renewal conditions. Results were highly similar for the two exposure designs with measured concentrations being approximately 80% of nominal. Under static conditions, concentrations declined somewhat through time. Measurements were highly repeatable within treatments. A recommendation emerged to conduct tests using a semi-static renewal design with 3,4-DCA in the future (OECD 2010). Tests with the remaining six compounds also followed this experimental design (OECD 2009; *Zebrafish Embryo Toxicity Test*, Standard Operation Procedure, SOP ZFET OECD V02.9, November 13th, 2009).

The objectives in the present study were to:

- Develop and apply suitable methods to verify stock and exposure solutions using compound-appropriate non-specific or specific methods;
- Verify stock solution concentrations for each of the six compounds in Phase 1b of the OECD validation program for the FET; and,
- Determine exposure concentrations in one representative laboratory (P&G).

2-GENERAL METHODS AND APPROACHES

2.1-Chemicals

The six compounds tested in Phase 1b are listed in Table 1 along with relevant physical-chemical parameters. Five of the six were organic compounds. Several of the compounds could be classified as "challenging chemicals" with relatively high Kow values (>4.0), low solubility (<10 mg/L) or moderately high volatility (> 10 Pascals-m^3/mole). Table 2 provides 2-dimensional structures and SMILES notation useful for modeling and chemical characterization.

2.2-Preparation of Stock Solutions

Stock solution preparation was outlined in the Phase 1b Trial Plan (Zebrafish Embryo Toxicity Test, Evaluation of transferability, intra- and interlaboratory reproducibility Trial Plan for Phase 1b – Testing of Six Chemicals, *TP_ZFET_OECD_1b_V01.1*, November 13th 2009) and is also given in the subsequent sections for each chemical.

2.3-Preparation of Exposure Solutions

Exposure solutions were prepared from stocks fresh each day of the test (i.e. every 24 hrs). Nominal exposure concentrations are given in Table 3. Exposure solutions were prepared as described in the Trial Plan. Wells of test plates were soaked with the appropriate exposure solution (material and concentrations) at least 24 hr in advance of initiating the definitive test. Sampling of wells for analytical verification was performed before and after renewal except for sodium chloride which utilized surrogate beakers to accommodate the probe for a conductivity meter.

2.4-Comparison of Aquatic Toxicity Estimates

Summarization of acute aquatic toxicity of all six chemicals in the FET tests was conducted in the P&G laboratories using regression models appropriate to the data structures. In this exercise, studies were summarized as 96-hr LC50s based on the effect (mortality) endpoints described in the SOP – coagulation of the egg, lack of somites, lack of tail detachment, and lack of heartbeat. The influence of measured versus nominal exposure concentrations using 96-hr EC50 determinations was made using the Trimmed Spearman-Karber method (Hamilton et al. 1977) and the Binomial Method (Stephan 1977, 1982).

3-METHODS AND RESULTS: Ethanol

Ethanol did not require stock solution preparation and was used directly in preparing test solutions as directed in the trial plan. Analyses were conducted within the P&G Central Product Safety laboratories.

3.1-TOC Method (Exposure and Stock Solution Concentration Determinations)

EtoH concentrations were determined by TOC analysis using a Shimadzu TOC-V Combustion Analyzer in the P&G Central Product Safety laboratories at Miami Valley Innovation Center (J. M. Rawlings). A 1000 mg/L EtOH standard was prepared daily. The calibration curve was prepared at 0, 20, 71.43, 125, 200, 250, and 333 mg/L. Check samples were also prepared daily at 20 and 333 mg/L and analyzed every 10-15 samples. Samples of exposure solutions were diluted to bring them into the range of the calibration curve. The arithmetic means of triplicate samples from all concentrations and times were determined. Final exposure concentrations were calculated as the geometric means of the measurements at each time point.

3.2-Analytical Results for Ethanol

Daily calibration curves based on normal linear regression analysis had slope, intercept, and r^2 that ranged from 2.394-2.904, (-4.458) – (- 10.72), and 0.9992-0.9998. Slight declines, roughly 5%, in ethanol were detected over each 24-hr period at each concentration (Table 4, Figure 1). Measured concentrations at 5.3, 8, 12, 18, and 27 g/L were 5.0, 7.3, 16, and 25 g/L (corresponding to 93.9, 91.4, 90.6, 91.6, and 92.8% of nominal, respectively).

4-METHODS AND RESULTS: Sodium Chloride

Analyses were conducted within the P&G Central Product Safety laboratories.

4.1-Specific Conductivity Method (Exposure and Stock Solution Concentration Determinations for NaCl)

Sodium chloride (NaCl) concentrations were determined in the P&G Central Product Safety laboratories at Miami Valley Innovation Center (J. M. Rawlings). Sodium chloride (50g) was dissolved in 1L of dilution water. The stock was stirred for 30 minutes at room temperature to ensure the sodium chloride was completely dissolved. The pH of the stock solution was adjusted to that of the dilution water (± 0.5) if needed. Stock solutions were kept at room temperature in a closed container because it is not subject to any degradative loss. The stock solution was stirred for 30 min prior to use to ensure uniform concentration of the substance.
A separate 50 g/L NaCl stock was prepared for calibrations. The stock was stirred for a minimum of 30 minutes prior to the preparation of the calibration standards. Standards were prepared at 0.75, 2.5, 7.5, 12.5, and 18.0 g/L in reconstituted water. The calibration curve was measured for specific conductance using a YSI 556 MPS at 0 h, the solutions were stored at room temperature and measured again at 96 hr. Results were averaged for the two time points and a linear regression for specific conductance calculated. NaCl concentrations on exposure samples were determined using specific conductance measurements on all new and old solutions at each time point.

4.2-Analytical Results for NaCl

Daily calibration curves had slope, intercept and r^2 that ranged from 1.6481-1.6770, 0.9764-0.9795, and 0.9994-0.9995. As would be expected for an inorganic salt, stock and exposure solutions were stable. Stock solutions at 50 g/L averaged 104.8% of nominal (±2.0% SD) for the three trial runs (Table 5). Measured concentrations at 1, 2, 4, 8, and 16 g/L were 0.94, 2.0, 4.1, 8.2 and 16/L corresponding to 94.5, 102.3, 103.6, 102.2, and 97.8% of nominal respectively (Table 6), with no losses during exchanges of test solutions (Fig. 2).

5-METHODS AND RESULTS: 2,3,6-Trimethyl phenol (TMP)

2,3,6-Trimethyl phenol (TMP) was analyzed by the P&G Trace Analytical Core at Mason Business Center (M. Karb, K. Wehmeyer).

5.1-HPLC/UV Method (Exposure and Stock Solution Sample TMP Concentrations)

Concentrations (µg/mL) of TMP in samples were determined by a reversed-phase high performance liquid chromatography method with ultraviolet detection (HPLC/UV). Samples were in Reconstituted Water (RW) consistent with the trial plan. Standard (STD) and Quality Control (QC) samples were prepared in Blended Water (BW from MVIC). Samples, STDs and QCs were analyzed directly by HPLC/UV. The nominal range of quantitation was 0.5 to 100 µg TMP/mL. Samples of TMP concentrations (µg/mL) in RW were determined by interpolation from a linear weighted (1/x) regression of STD TMP concentrations in BW by instrument response factors (peak area for UV absorbance at λ = 275 nm). For the purpose of quantitation, the BW and RW matrices were considered to be equivalent (full details archived at P&G as Method No.: HCL13857_2,3,6-Trimethylphenol in Blended Water_ Revision No.: 0) .

Samples were shipped at room temperature. All water samples were analyzed on the day they were received, following mixing of the sample, and transfer to autosampler vials. STD and QC samples derived from separate compound stock weighings and were prepared fresh on the days of study sample receipt. A STD curve was run at the beginning and end of the HPLC/UV run, and samples were evenly interspersed with QC samples throughout the run to monitor any bias in results. All exposure solution samples were analyzed using the STD curve. In the case of stock solutions, analysis was done either using the complete STD curve as described above, or by a single concentration point STD calibration (80 µg/mL), with an additional QC point from a separate weighing also analyzed. The stock solution samples were diluted to a nominal concentration of 80 µg/mL, whether a full standard curve or a single point calibration was used. The single point calibration was done on days in which only stock solutions and not exposure solutions were received. In the case of the single point calibrations, duplicate dilutions were performed for STDs and QCs as a quality check, and stock solution samples were diluted in duplicate to be the same nominal concentration as the STDs.

In the case of stock solutions, samples were analyzed using a single point calibration, and the concentration of TMP in the samples was calculated using Equation 1:

$$\text{TMP conc. (µg/mL)} = \frac{\text{Mean Study Specimen Peak Area}}{\text{Mean STD Peak Area}} \times 250\,\text{(µg/mL)} \qquad \text{Equation 1}$$

The final TMP concentration result for each sample was calculated as the mean result of both aliquots, expressed as µg TMP/mL (or mg/L). Data was provided for this and all other compounds to the P&G Aquatic Toxicology Laboratory in a form suitable for use in EXCEL.

Standards prepared at theoretical concentrations of 0.5, 1, 2, 5, 10, 20, 50 and 100 mg/L possessed %RE (Relative Error, nominal versus found) in the range of +/- 8-10%. Additional QC samples prepared in quadruplicate at 0.8, 8 and 80 mg/L had %RSDs of 8.5, 8.3, and 8.0%, respectively.

5.2 - Preparation of TMP Stock Solutions and Exposure Solutions for tests at P&G MVIC

The long term stability of this compound was not known under storage conditions; therefore, a new stock solution was always prepared for each run. Approximately 250 mg 2,3,6-trimethylphenol was dissolved in 1L of dilution water. Stock solutions were stirred in a closed, light proof vessel for at least 24 h at room temperature to ensure the TMP was completely dissolved. Solution pH was adjusted to the pH of the dilution water (± 0.5) if needed. The stock solution was kept refrigerated in the dark (1-8°C) during each run. Prior to use of the stock, the solution was stirred at room temperature for 30 min to ensure uniform concentration of the substance. Samples of each stock solution were prepared for concentration confirmation, stored under refrigerated conditions and shipped to the P&G TAC laboratory.

5.3 - Analytical Results for TMP

The average of three stock solution samples was 103.1% of nominal (250 mg/L target, average of 257.7 mg/L measured) indicating the stock was accurately prepared (Table 5).
Geometric mean measured concentrations throughout the test at 8, 12, 18, 27 and 40.5 mg/L were 8.5, 12.7, 18.9, 28.3, and 41.7 mg/L (Table 7). These represent 102.8-106.0% of nominal. Slight losses (1.7-10.2%) over the 24-hr renewal period were observed across all concentrations (Figure 3).

6-METHODS AND RESULTS: 6-Methyl-5-hepten-2-one (MHO)

6-Methyl-5-hepten-2-one (MHO) was analyzed by the P&G Trace Analytical Core at Mason Business Center (M. Karb, K. Wehmeyer).

6.1 - HPLC/MS/MS Method (Study Sample MHO Exposure Concentration Determinations)

Concentrations of MHO, and its chemical internal standard (IS), 5-methyl-2-hepten-4-one (5M), were determined in Blended Water (BW) study exposure samples by a reversed-phase high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) method operating under multiple reaction monitoring (MRM) conditions (full details archived at P&G as Method # HCL_13855_6-Methyl-5-Hepten-2-One in Blended Water_ Revision No.: 0).

Exposure solution samples with nominal test concentrations ranging from 25 to 208 µg MHO/mL of BW were received by the Trace Analytical Core (TAC) on dry ice. Exposure solutions were analyzed by TAC on the day they were received as follows. Samples were thawed and mixed. Specimens were diluted 1:100 by adding 990 µL of BW plus 10 µL of each study sample to autosampler vials with silicone septum caps. Blank samples were diluted 1:4 because there was not a full 1000 µL to sample directly.

MHO STD (W-STD) and working QC (W-QC) samples derived from separate compound stock weighings were prepared in BW at concentrations ranging from 50 to 10000 ng MHO/mL. An aliquot (1000 µL) of each W-STD and W-QC was added to each autosampler vial. 5M IS (10 µL) was added to each W-STD and W-QC sample, plus diluted exposure solution samples in autosampler vials, followed by immediate capping and mixing. A STD curve was run at the beginning and end of the HPLC/MS/MS run, and exposure solution samples were evenly interspersed with QC samples throughout the run to monitor any bias in results.

Diluted MHO exposure solution sample concentrations (ng/mL) were determined by interpolation from a quadratic weighted (1/x2) regression of STD MHO concentrations by HPLC/MS/MS instrument response factors (peak area MHO/peak area 5M). Final concentration results in the original BW matrix were then

calculated by multiplying diluted sample MHO concentrations by the overall dilution factor applied to the sample (100), followed by conversion from ng/mL to µg/mL.

6.2 - HPLC/UV Method (Stock Solution Sample MHO Concentrations)

Stock STD and QC solutions (1 mg MHO/mL BW) were prepared from separate weighings. Study stock solution samples, also at a nominal concentration of 1 mg MHO/mL BW, were received shipped on ice packs (~4°C), and analyzed on the day they were received. The STD and QC Stocks, and the stock solution samples were each diluted to a nominal concentration of 10 µg/mL in BW Diluent (n=2 each). Stock solution samples were analyzed on the day received.

Diluted STD, QC and stock solution samples were analyzed by reversed-phase HPLCUV with a C18 column, using a Waters Acquity HPLC/UV system (monitored l=200 nm). Each aliquot of STDs, QCs and study samples was injected in triplicate, and a single point calibration was made using the overall mean peak area of both STD aliquots. The concentration of MHO in each QC and sample was calculated using Equation 2:

$$\text{MHO conc. (µg/mL)} = \frac{\text{Mean Study Specimen Peak Area}}{\text{Mean STD Peak Area}} \times 1000 \text{ (µg/mL)} \quad \text{Equation 2}$$

The final MHO concentration result for each Study Specimen Stock was calculated as the overall mean result for both aliquots, expressed as µg MHO/mL BW.

6.3 - Preparation of MHO Stock Solutions and Exposure Solutions for Tests at P&G MVIC

Approximately 1000 mg 6-Methyl-5-heptene-2-one was dissolved in 1L of dilution water. The substance is a liquid, hence, correction for density at 0.852 g/cm³ was used to disperse the neat material. Ultimately 1000 mg corresponds to 1174 µL of the substance. Stock solutions were stirred in a closed, light proof vessel for 30 minutes at room temperature to ensure that the 6-methyl-5-heptene-2-one is completely dissolved. The pH of the stock solution was adjusted to the pH of the dilution water (± 0.5) if needed and the stock solution was kept refrigerated in the dark (1-8°C) during a single run. Prior to use of the stock, the solution was stirred at room temperature for 30 min to ensure uniform concentration of the substance. Samples of each stock solution were prepared for concentration confirmation, stored under refrigerated conditions and shipped to the P&G TAC laboratory.

6.4 - Analytical Results for MHO

The average of three replicate stock samples was 98.6% of nominal (1000 mg/L target, average 985.7 mg/L measured) indicating the stock was accurately prepared (Table 5).
Geometric mean measured concentrations throughout the test at 25, 42.5, 72.25, 122.825, and 208.03 mg/L were 18.7, 33.0, 61.7, 114, and 154 mg/L, respectively (Table 8). These levels were 74.0-92.9% of nominal. Substantial losses over the 24-hr renewal period were observed across all concentrations (Figure 4).

7 - METHODS AND RESULTS: Dibutyl maleate (DM)

Dibuytl maleate (DM) was analyzed by the P&G Trace Analytical Core at Mason Business Center (M. Karb, K. Wehmeyer).

7.1 - HPLC/MS/MS Method (Study Sample DM Exposure Concentration Determinations)

Concentrations of DM, and its stable isotope-labeled internal standard, Dibutyl Maleate-D_{20} (DM-D_{20}), were determined in Reconstituted (RW) samples by a reversed-phase high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) method operating under multiple reaction monitoring (MRM) conditions (full details archived at P&G as Method # HCL_13858_Dibutyl Maleate in Blended Water Revision No.: 0).

Reconstituted water (RW) exposure solution samples were diluted 1:1 (v:v) with MeOH at the CPS Environmental study site prior to receipt by the Trace Analytical Core (TAC), with interim storage at ~4°C in amber vials with Teflon-lined caps covered with parafilm. All samples were analyzed by Analytical on the day they were received.

Upon receipt by TAC, exposure solutions with nominal test concentrations ranging from 0.25 to 4.0 mg DM/L of RW (nominally 0.125 to 2.0 mg/L in 50:50 MeOH:RW as received) were diluted an additional 50-fold with 50:50 Blended Water:MeOH (Diluent) in 96-well plates by combining 8 µL of each sample with 392 µL of Diluent (nominal concentration ranges of 0.0025 to 0.04 mg/L in diluent after a total 100-fold dilution (a 2-fold dilution at the study site with an additional 50-fold dilution by TAC, not corrected for the small differences in specific gravity of the mixtures of MeOH and water, versus water alone). Exposure solutions with a nominal test concentration of 0 mg/L of RW (also received in a 1:1 RW:MeOH matrix) were added directly to 96-well plates (400 µL) without further dilution.

Standard (STD) and QC samples derived from separate compound stock weighings were prepared in diluent at concentrations ranging from 0.00025 to 0.200 mg/L (0.25 to 200 ng/mL) and added to 96 well plates (400 µL). DM-D_{20} internal standard was added to both diluted and undiluted stock samples, and STD and QC samples. A STD curve was run at the beginning and end of the HPLC/MS/MS run, and stock samples were evenly interspersed with QC samples throughout the run to monitor any bias in results.

Samples of DM from FET tests (ng/mL) were determined by interpolation from a quadratic weighted ($1/x^2$) regression of STD DM concentrations by HPLC/MS/MS instrument response factors (peak area DM/peak area DM-D_{20}). Final concentration results in the original RW matrix were then calculated by multiplying specimen DM concentrations determined by HPLC/MS/MS analysis by the overall dilution factor applied to the sample (nominal 0 mg/L exposure solution samples were multiplied by a factor of 2 and nominal 0.25 to 4.0 mg/L exposure solution samples were multiplied by 100).

7.2 - HPLC/UV Method (Stock Solution Sample DM Concentration Determinations)

Stock standard (STD) and QC solutions (5 mg DM/mL MeOH) were prepared from separate weighings. The STD and QC stocks were each diluted to 25 µg/mL in Diluent (n=2 each).

Stock solution samples were received in duplicate (nominal 50 mg/L = 50 µg/mL in RW which equates to an effective nominal concentration of 25 µg/mL in 50:50 RW:MeOH following a 1:1 dilution with MeOH at the Study Site). Stock solution samples were added to autosampler vials in duplicate (one sampling right after mixing and a second sampling after time had passed to test for any lack of sample homogeneity). Stock solutions were analyzed on the day received.

STD, QC and Study stock samples were analyzed by reversed-phase HPLC-UV with a C18 column, using a Waters Acquity HPLC/UV system (monitored λ=195 nm). Each aliquot of STDs, QCs and stock solution samples was injected in triplicate or quadruplicate, and a single point calibration was made using the overall mean peak area of both STD aliquots. The concentration of DM in each QC and study Specimen was calculated using Equation 3:

$$\text{DM conc. (µg/mL)} = \frac{\text{Mean Sample Peak Area}}{\text{Mean STD Peak Area}} \times 50 \qquad \text{Equation 3}$$

The final DM concentration result for each stock solution sample was calculated as the overall mean result for both aliquots, expressed as µg DM/mL RW.

Standards prepared at theoretical concentrations of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 200 mg/L possessed %RE (Relative Error, nominal versus found) in the range of +/-5-9% (average for RE all samples 0.7%) . Additional QC samples prepared in quadruplicate at 0.75, 7.5 and 75 mg/L had %RSDs of 1.8, 2.9, and 1.8%, respectively.

7.3 - Preparation of DM Stock Solutions and Exposure Solutions for Tests at P&G MVIC

Dibutyl maleate (50 mg) was dissolved in 1L of dilution water. The substance is a liquid and the density is near 1.0 (0.994 g/cm³) thus 50 mg corresponded to 50 µL of the test substance. The DM stock was stirred in a closed, light proof vessel for 30 minutes at room temperature to ensure that the material was completely dissolved. Solution pH was adjusted to that of the dilution water (± 0.5). DM stocks were kept refrigerated in the dark (1-8°C) during each run. Before use, the stock solution was stirred at room temperature for 30 min to ensure uniform concentration of the substance. Two samples from each stock solution were preserved 1:1 with methanol and stored in the refrigerator.

7.4 - Analytical Results for DM

Three replicate stocks solutions of DM (50 mg/L) were measured at an average of 51.9 mg/L or 103.1% of nominal (Table 5).
Geometric mean measured concentrations throughout the test at 0.25, 0.5, 1.0, 2.0, and 4.0 mg/L were 0.14, 0.31, 0.59, 1.4, and 2.5 mg/L, respectively (Table 9). These represent losses of 57.3-68.2% of nominal. Losses occurred over the 24-hr renewal period and were relatively exposure dependent with the greatest losses at the lowest concentrations (0.25-0.5 mg/L nominal). The overall range of losses in the 24-hr period was -10.5 to -28.0% (Figure 5).

8 - METHODS AND RESULTS: Triclosan (TCS)

Triclosan (TCS) was analyzed by the P&G Trace Analytical Core at Mason Business Center (M. Karb, K. Wehmeyer).

8.1 - HPLC/MS/MS Method (Study Samples TCS Exposure Concentration Determinations)

Concentrations of TCS, and its stable isotope-labeled internal standard, ^{13}C6-Triclosan (^{13}C6-TCS), were determined in Reconstituted (RW) Study Specimen samples by a reversed-phase high performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) method operating under multiple reaction monitoring (MRM) conditions (full details archived at P&G as Method # HCL_13859_Triclosan in Reconstituted Water Revision No.: 0).
RW exposure solution samples (containing RW with 0.1% Ethanol (EtOH)) were diluted 1:1 (v:v) with MeOH at the CPS Environmental study site prior to receipt by the Trace Analytical Core (TAC), resulting in a matrix consisting of 50:49.95:0.05 MeOH:RW:EtOH (RW Diluent), with interim storage at ~4°C in amber vials with Teflon-lined caps covered with parafilm. Exposure solution samples with nominal test concentrations ranging from 75 to 1200 ng Triclosan/mL of RW (nominally 37.5 to 600 ng/mL in RW Diluent following dilution with MeOH) were received by TAC. Samples were not corrected for the small differences in specific gravity of the mixtures of MeOH and water, versus water alone. All samples were analyzed by TAC on the day they were received.
STD and QC samples derived from separate compound stock weighings were prepared in a 50:49.95:0.05 MeOH:Blended Water (BW):EtOH matrix (BW diluent) at concentrations ranging from 5 to 1000 ng TCS/mL to approximate the RW diluent (RW diluent and BW diluent matrices cross-validated (See Method Reference)). ^{13}C6-TCS internal standard (20 µL) was combined with 400 µL of STD and QC samples, and diluted Study Specimens. A STD curve was run at the beginning and end of the

HPLC/MS/MS run, and samples were evenly interspersed with QC samples throughout the run to monitor any bias in results.
Sample TCS concentrations (ng/mL) were determined by interpolation from a quadratic weighted (1/x2) regression of STD TCS concentrations by HPLC/MS/MS instrument response factors (peak area TCS/peak area ^{13}C6-TCS). Final concentration results in the original RW matrix were then calculated by multiplying sample Triclosan concentrations determined by HPLC/MS/MS analysis by the overall dilution factor applied to the sample (2).

8.2 - HPLC/UV Method (Stock Solution Sample TCS Concentrations)

Stock STD and QC solutions (1 mg TCS/mL MeOH) were prepared from separate weighings. The STD and QC stocks were each diluted to 10 µg/mL in BW diluent (n=2 each). Stock solution samples were received in duplicate (nominal 1200 µg/mL in EtOH which equates to an effective nominal concentration of 600 µg/mL in 50:50 EtOH:MeOH, following a 1:1 dilution with MeOH at the Study Site). Stock solution samples were added to autosampler vials in duplicate and diluted by a factor of 120 or 240 with BW Diluent, to a nominal concentration of 10 µg/mL or 5 µg/mL, respectively. Stock solution samples were analyzed on the day received.
Diluted STD, QC and stock solution samples were analyzed by reversed-phase HPLC/UV with a C18 column, using a Waters Acquity HPLC/UV system (monitored l=230 nm). Each aliquot of STDs, QCs and Study Specimens was injected in triplicate, and a single point calibration was made using the overall mean peak area of both STD aliquots. The concentration of TCS in each QC and study stock solution samples was calculated using Equation 4:

$$\text{TCS conc. (µg/mL)} = \frac{\text{Mean Sample Peak Area}}{\text{Mean STD Peak Area}} \times 10\,\text{µg/mL} \times \text{sampleDF} \quad \text{Equation 4}$$

The final Triclosan concentration result for each stock solution sample was calculated as the overall mean result for both aliquots, expressed as µg Triclosan/mL EtOH.

8.3 - Preparation of Stock Solutions and Exposure Solutions for Tests at P&G MVIC

Approximately 120 mg TCS was dissolved in 100 mL of ethanol (200 proof, ACS/USP grade). The solution was stirred in a closed, light proof vessel for 30 minutes at room temperature to ensure the TCS is completely dissolved. Adjustment of pH was not needed. The stock solution was suitable to be kept in the refrigerator (1-8°C) for several weeks. Prior to use of the stock, the solution was stirred at room temperature for 30 min to ensure uniform concentration of the substance. Two samples from each stock solution were stored at 1-8°C until analysis.

8.4 - Analytical Results for TCS

Three TCS stocks solutions (1200 mg/L) were measured at an average of 1202.3 mg/L or 100.2% of nominal (Table 5).
Geometric mean measured concentrations throughout the test at 0.075, 0.15, 0.3, 0.6, and 1.2 mg/L were 0.072, 0.14, 0.29, 0.61, and 1.2 mg/L, respectively (Table 10). These represent measurements that were 91.1-100.9% of nominal. Slight losses occurred over the 24-hr renewal period and were relatively exposure dependent with the greatest losses at the lowest concentrations (0.075-0.15 mg/L nominal). The overall range of losses in the 24-hr period was -21.2 to -7.2% (Figure 6).

9 - ADDITIONAL RESULTS

The studies on the six compounds described in this report span a wide range of expected acute aquatic toxicities ranging from very low (> 1 g/L) to highly toxic (< 1 mg/L). Analytical confirmation of exposure has important consequences for LC50 or EC50 determinations. According to OECD technical guidelines (e.g. OECD 1992, 2004) if the measured exposure concentrations depart from nominal by ±20% then measured concentrations should be used in calculating effect concentrations. Initial measured concentrations were uniformly within 10% of nominal in all studies except for 6-methyl-5-hepten-2-one (MHO). In the case of MHO, initial measured concentrations in some exposures (122.825-208.03 mg/L) were >100% of nominal. Evidence from these studies suggests that losses in the dibutyl maleate would clearly result in a need to perform analytical measurements and calculate effect concentrations accordingly (Table 9) and that while MHO underwent substantial losses between renewals, the geometric mean measured concentrations for all the exposures were 75-93% of nominal (Table 8). Ethanol, sodium chloride, 2,3,6-trimethylphenol and triclosan all had measured exposures within 20% of the nominal. Collectively, effects of measured versus nominal concentrations on the LC50 should be relatively small for these compounds (Tables 4, 6, 7, and 10, respectively).
As another means to evaluate the importance of quantifying variations from the nominal concentration when using measured concentrations in determining the effect values, we compared 96-hr LC50s using nominal compared to those derived using measured concentrations. Of all the compounds tested, dibutyl maleate had the greatest difference in nominal versus measured 96-hr LC50s declining from 0.70 ± 0.012 (n=3) mg/L to 0.42 mg/L (Table 10), a change of -39.5%. For 6-methyl-5-hepten-2-one, the decline was less substantial (-17.1%), but this was offset somewhat by initial measured concentrations being *above the nominal* while the renewals were far below the nominal. In an attempt to visualize the nominal versus measured LC50 differences the changes were plotted against the Henry's Law Constant (as a measure of volatility) and the log Kow as a measure of hydrophobicity (Figure 7). Kow also serves as a surrogate for sorptivity as well. While the influence of physical-chemical properties upon changes in the LC50 are not fully obvious, the more sorptive and volatile compounds do appear the most problematic (note that degradation here is not discounted but is also a potential contributor).

10 -OVERALL CONCLUSIONS

1. Clearly by these studies, determination of exposure concentrations in the FET can be accomplished by modern analytical methods, even when very low sample volumes and highly toxic substances are involved.
2. Non-specific methods may be useful under certain conditions and should be considered as options when possible.
3. Stock solutions for all tested chemicals were consistently and reliably prepared. Departures from nominal were uniformly <5%.
4. Analytical confirmation of exposure is likely not essential for every compound and every study. Some FET tests may provide reliable LC50 determinations under static conditions (versus semi-static) when exposures can be maintained.
5. The most challenging compounds were characterized by combinations of low solubility, moderate to higher hydrophobicity, and being semi-volatile.
6. Analytical confirmation of exposure for challenging compounds was necessary and is reflected in lower 96-hr LC50 estimates for at least two compounds.

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Table 1. Summary of physical chemical properties of compounds entered into Zebrafish Fish Embryo Testing, Phase 1b.

Compound	**CASNO**	**MW**	**Log Kow**	**HLC (Pas-m3/mole)**	**Solubility (mg/L)**	**Expected Toxicity Range**	**Chemical Purity (%)**
Ethanol	64-17-5	46.07	-0.31	0.574	1×10^6	Nontoxic	>99.9
Sodium chloride	7647-14-5	58.44	N/A	N/A	359000	Nontoxic	100
2,3,6-Trimethyl phenol	2416-94-6	136.2	3.15	0.399	1580	Moderately Toxic	99.6
6-methyl-5-hepten-2-one	110-93-0	126.2	2.06	21.5	4364.1	Moderately Toxic	96.0
Dibutyl maleate	105-76-0	228.29	4.16	0.0768	8.709	Toxic	97.8
Triclosan	3380-34-5	289.55	4.76	0.00051	4.621	Very toxic	99.7

Table 2. SMILES notation and structures associated with the compounds

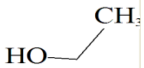
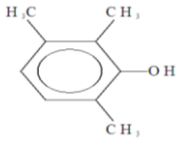
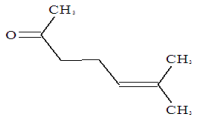
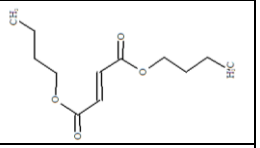
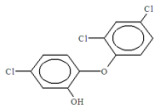
Compound	**SMILES Notation**	**Structure**
Ethanol	OCC	
Sodium chloride	N/A	N/A
2,3,6-Trimethyl phenol	Oc1c(ccc(c1C)C)C	
6-methyl-5-hepten-2-one	O=C(CCC=C(C)C)C	
Dibutyl maleate	O=C(OCCCC)C=CC(=O)OCCCC	
Triclosan	O(c(c(O)cc(c1)Cl)c1)c(c(cc(c2)Cl)Cl)c2	

Table 3. Dates of studies and nominal exposure concentrations used in each.

Compound	Nominal Exposure Concentrations	Date Z-FET Study	Study for Which Analytical

	(mg/L)	was Initiated	Verification of Exposure was Determined
Ethanol	0, 5300, 8000, 12000, 18000, 27000	19 Apr 2010 3 May 2010 24 May 2010	24 May 2010
Sodium chloride	0, 1000, 2000, 4000, 8000, 16000	25 Jan 2010 22 Feb 2010 8 Mar 2010	25 Jan 2010
2,3,6-Trimethyl phenol	0, 8.0, 12.0, 18.0, 27.0, 40.5	8 Feb 2010 22 Feb 2010 22 Mar 2010	22 Feb 2010
6-methyl-5-hepten-2-one	0, 25.0, 42.5, 72.25, 122.825, 208.03	19 Apr 2010 3 May 2010 24 May 2010	3 May 2010
Dibutyl maleate	0, 0.25, 0.50, 1.0, 2.0, 4.0	8 Feb 2010 8 Mar 2010 22 Mar 2010	8 Mar 2010
Triclosan	0, 0.075, 0.15, 0.30, 0.60, 1.2	19 Apr 2010 3 May 2010 24 May 2010	19 Apr 2010

Table 4. Summary of measurements of ethanol concentrations during the conduct of a semi-static, 96-hr FET.

	Geometric mean (g/L)			% of nominal		
Nominal	New	Old	Combined	New	Old	Combined
5.3	5.09	4.87	4.98	96.0	91.8	93.9
8.0	7.40	7.24	7.32	92.4	90.5	91.4
12	11.1	10.6	10.9	92.7	88.4	90.6
18	16.9	16.1	16.5	93.8	89.4	91.6
27	25.6	24.5	25.1	94.9	90.8	92.8

Table 5. Summary of stock solution measurements for the 6 compounds used in this study.

	n	Nominal Stock Solution (mg/L)	Measured Stock Solution (mg/L)		% of Nominal	
			Average	Stdev	Average	Stdev
Ethanol				N/A		N/A
NaCl	3	50000	52400.0	1000.0	104.8	2.0
2,3,6-trimethylphenol	3	250	257.7	12.5	103.1	5.0
6-methyl-5-hepten-2-one	3	1000	985.7	12.3	98.6	1.2
6-methyl-5-hepten-2-one	3	1200	1202.3	2.1	100.2	0.2
Dibutyl maleate	3	50	51.9	2.1	103.9	4.3
Triclosan	3	1200	1202.3	2.1	100.2	0.2

Table 6. Summary of measurements of NaCl concentrations during the conduct of a semi-static, 96-hr FET.

	Geometric mean (g/L)			% of nominal		
Nominal	New	Old	Combined	New	Old	Combined
1	0.947	0.943	0.945	94.7	94.3	94.5
2	2.046	2.045	2.045	102.3	102.2	102.3
4	4.144	4.140	4.142	103.6	103.5	103.6
8	8.177	8.177	8.177	102.2	102.2	102.2
16	15.648	15.643	15.646	97.8	97.8	97.8

Table 7. Summary of measurements of 2,3,6-Trimethylphenol concentrations during the conduct of a semi-static, 96-hr FET.

	Geometric mean (mg/L)			% of nominal		
Nominal	New	Old	Combined	New	Old	Combined
8.00	8.58	8.45	8.48	107.3	105.6	106.0
12.0	12.9	12.4	12.7	107.9	103.7	105.8
18.0	19.1	18.6	18.9	106.2	103.5	104.8
27.0	28.8	27.7	28.3	106.8	102.5	104.6
40.5	43.8	39.6	41.7	108.1	97.9	102.8

Table 8. Summary of measurements of 6-methyl-5-hepten-2-one concentrations during the conduct of a semi-static, 96-hr FET.

	Geometric mean (mg/L)			% of nominal		
Nominal	New	Old	Combined	New	Old	Combined
25.0	23.5	14.8	18.7	94.2	59.1	74.6
42.5	43.4	25.1	33.0	102.1	59.2	77.7
72.25	78.4	48.6	61.7	108.5	67.2	85.4
122.825	149	87.6	114	121.1	71.3	92.9
208.03	244	96.9	154	117.5	46.6	74.0

Table 9. Summary of measurements of dibutyl maleate concentrations during the conduct of a semi-static, 96-hr FET.

	Geometric mean (mg/L)			% of nominal		
Nominal	New	Old	Combined	New	Old	Combined
0.25	0.18	0.11	0.14	72.1	45.6	57.3
0.50	0.39	0.25	0.31	78.4	50.4	62.8
1.0	0.64	0.53	0.59	63.8	53.3	59.1
2.0	1.5	1.2	1.4	74.0	60.5	68.2
4.0	2.8	2.3	2.5	71.0	56.8	63.5

Table 10. Summary of measurements of Triclosan concentrations during the conduct of a semi-static, 96-hr FET.

	Geometric mean (mg/L)			% of nominal		
Nominal	New	Old	Combined	New	Old	Combined
0.075	0.080	0.064	0.072	107.1	85.9	96.0
0.15	0.15	0.12	0.14	101.7	81.6	91.1
0.30	0.31	0.27	0.29	104.9	88.9	96.6
0.60	0.63	0.58	0.61	104.9	97.0	100.9
1.2	1.2	1.1	1.2	102.2	95.1	98.6

Table 11. Comparison of 96-hr LC50s calculated based on nominal and measured exposure concentrations.

		Nominal LC50			Nominal LC50			Measured LC50					% Change in the Nominal LC50
Compound	Run	LC50 (mg/L)	LCL (mg/L)	UCL (mg/L)	Mean (mg/L)	STDEV (mg/L)	% CV	LC50 (mg/L)	LCL (mg/L)	UCL (mg/L)	LC50 Method	Auto Trim	
NaCl	1	4600	3900	5300	5800	1100	18.50%	4700	4000	5500	TSK	2.50%	2.10%
	2	6600	5.800	7600							TSK	1.70%	
	3	6100	5500	6700							TSK	0.00%	
DM	1	0.7	0.68	0.72	0.7	0.012	1.80%				TSK	2.50%	
	2	0.71	0.5	1				0.42	0.41	0.43	Binomial	NA	-39.50%
	3	0.68	0.64	0.73							TSK	0.00%	
TMP	1	15.9	14.8	17.1	22.6	5.8	25.80%				TSK	0.00%	
	2	26.8	22.7	31.5				27.9	23.8	32.8	TSK	10.30%	4.30%
	3	25.1	22.7	27.6							TSK	0.00%	
Ethanol	1	13278	12224	14424	13333	814	6.10%				TSK	0.00%	
	2	14174	13460	14927							TSK	0.00%	
	3	12548	11486	13708				11597	10576	12717	TSK	0.00%	-7.60%
TCS	1	0.36	0.31	0.41	0.34	0.018	5.50%	0.35	0.3	0.4	TSK	0.00%	-1.90%
	2	0.34	0.29	0.39							TSK	0.00%	
	3	0.32	0.28	0.38							TSK	0.00%	
MHO	1	165	155	176	160	4.6	2.90%				TSK	15.00%	
	2	160	125	205				132	105	167	TSK	20.00%	-17.10%
	3	156	142	172							TSK	5.00%	

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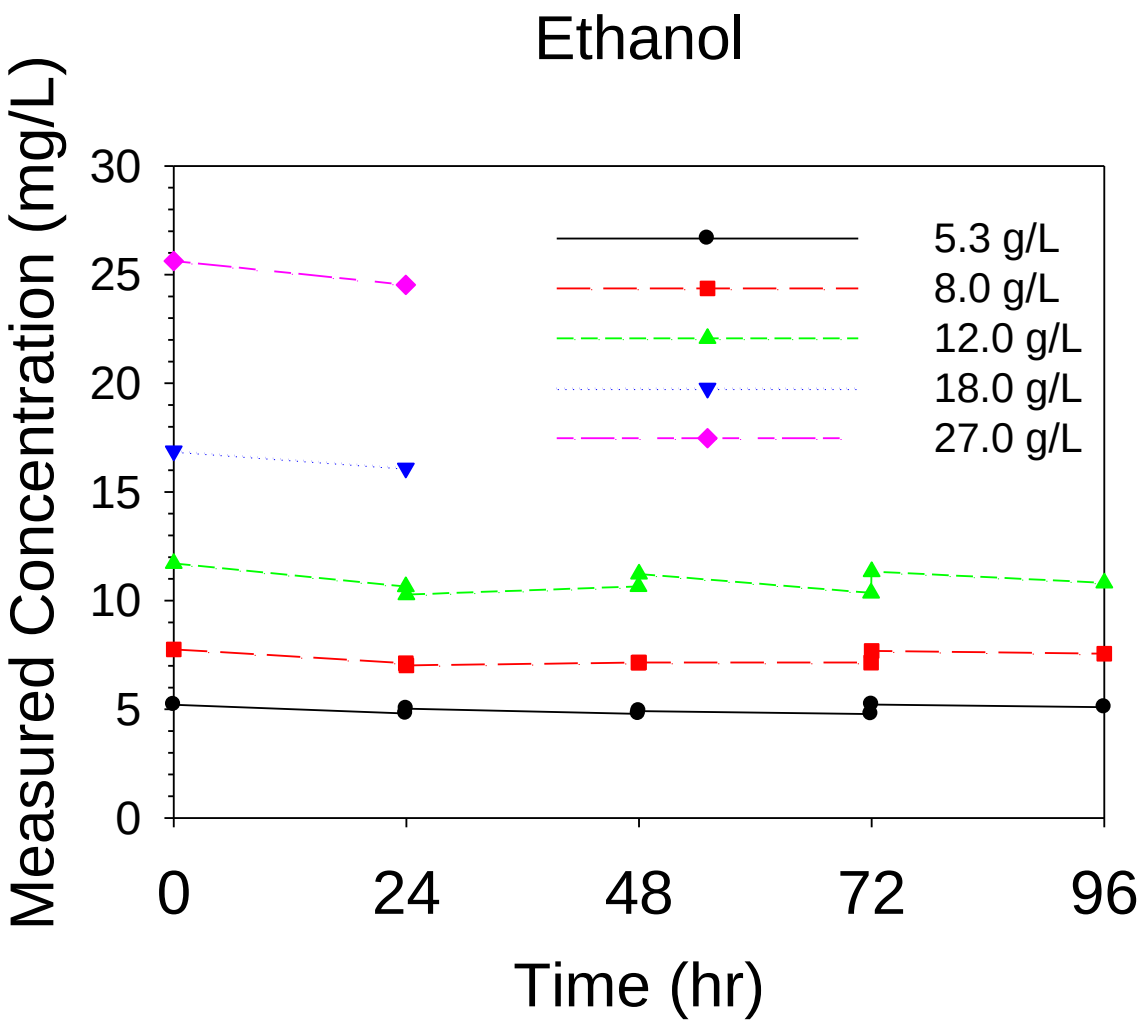

Figure 1. Pattern of measured ethanol concentrations in the FET using a 24-hr renewal, semi-static design.

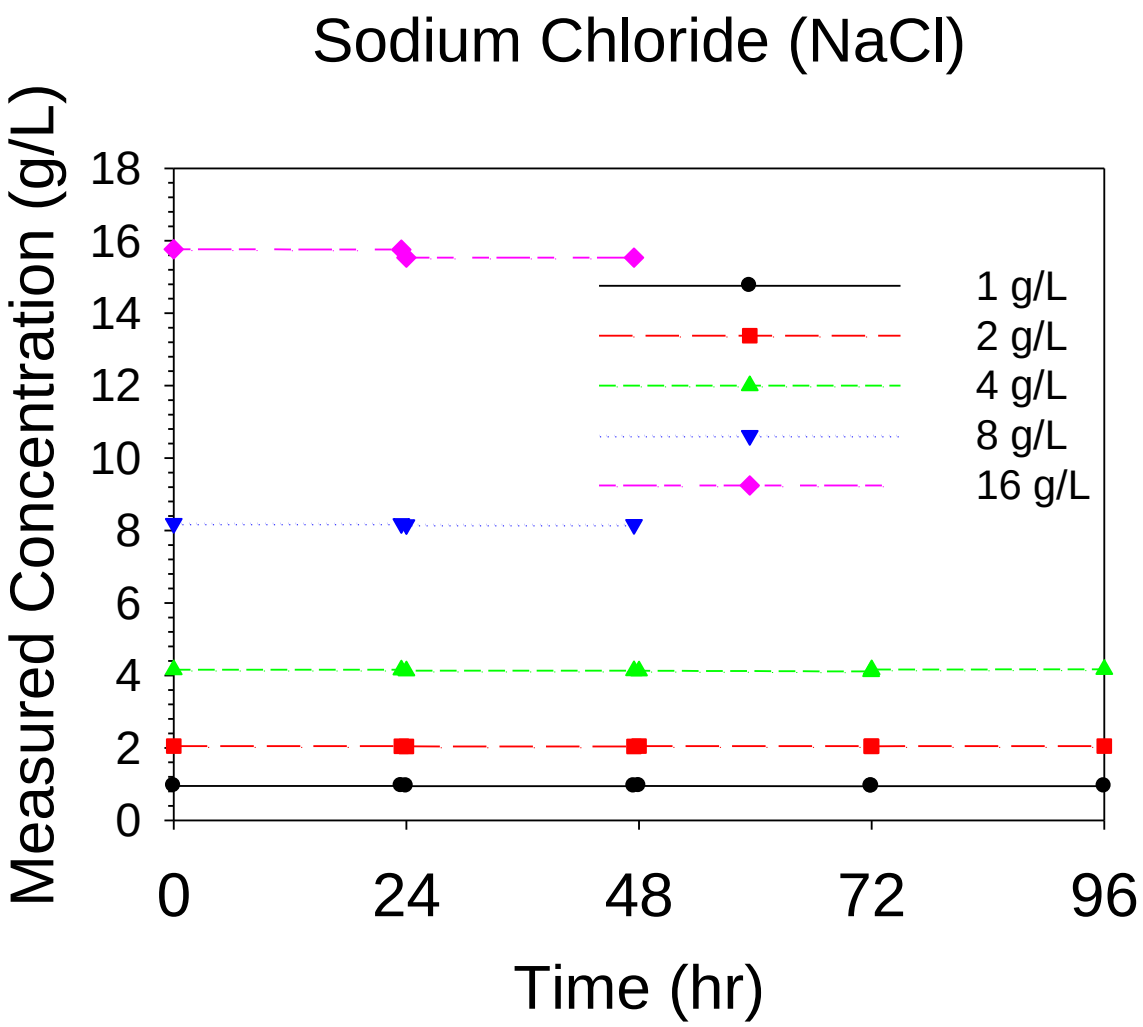

Figure 2. Pattern of measured sodium chloride concentrations in the FET using a 24-hr renewal, semi-static design.

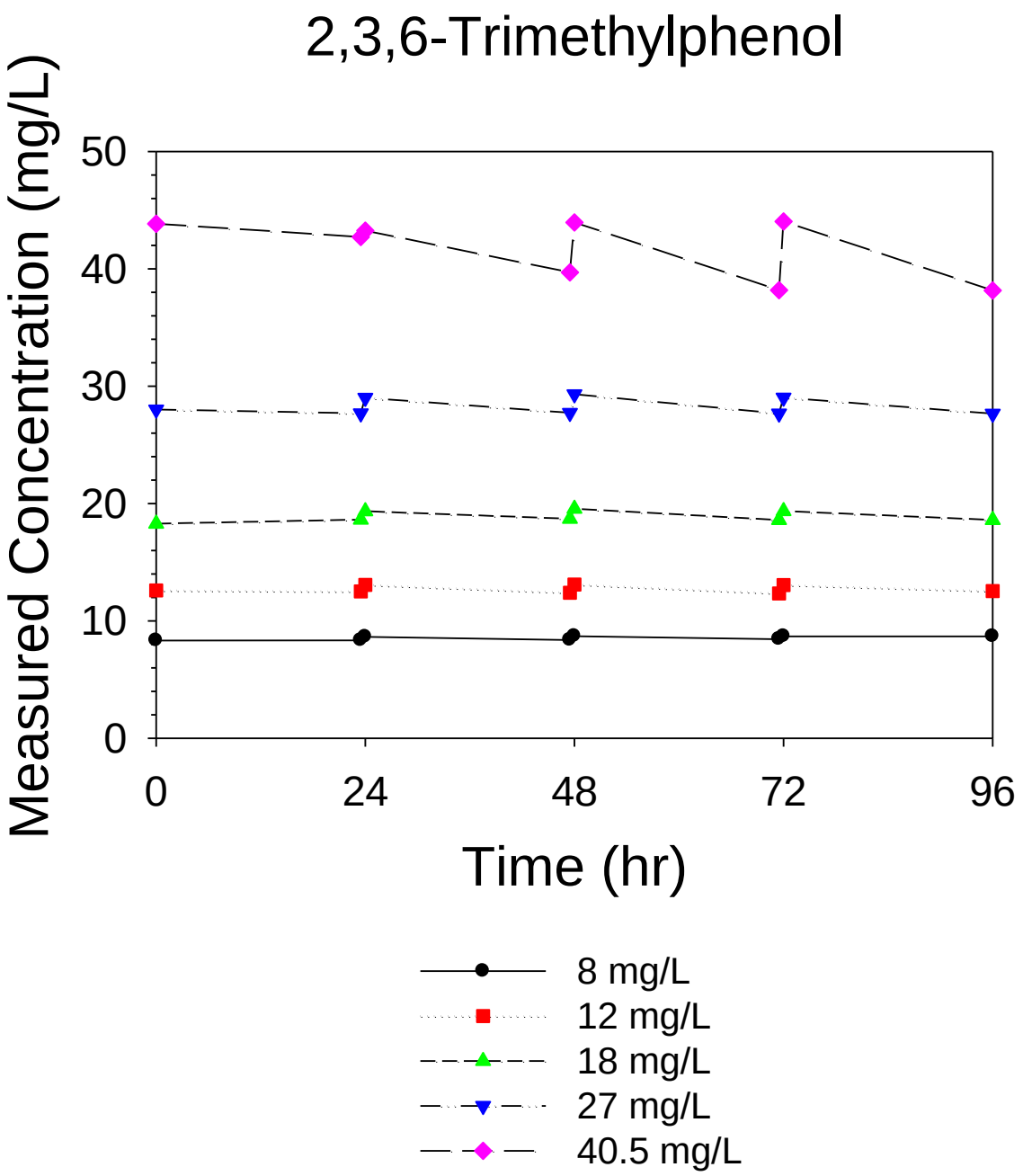

Figure 3. Pattern of measured 2,3,6-trimethylphenol concentrations in the FET using a 24-hr renewal, semi-static design.

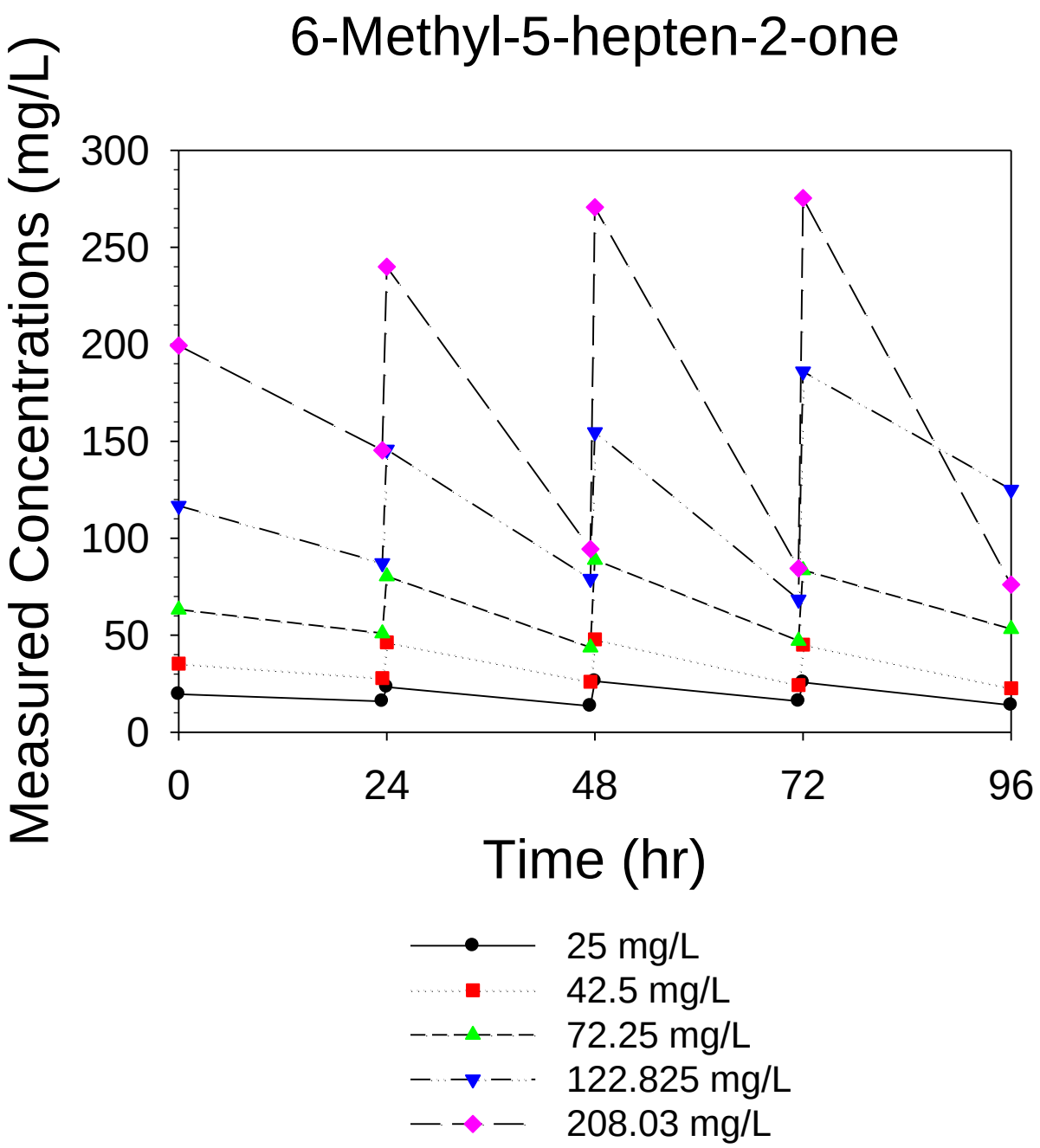

Figure 4. Pattern of measured 6-methyl-5-hepten-2-one concentrations in the FET using a 24-hr renewal, semi-static design.

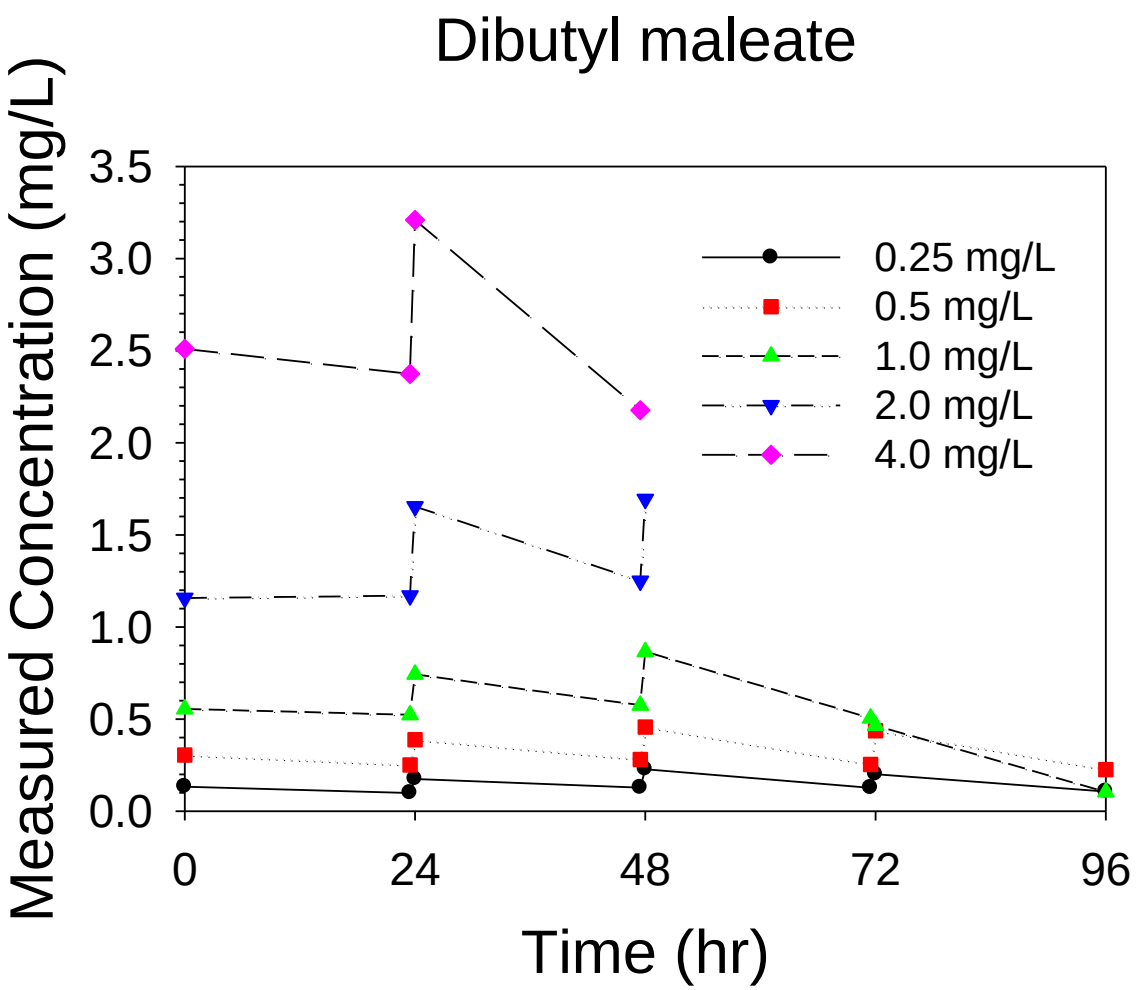

Figure 5. Pattern of measured dibutyl maleate concentrations in the FET using a 24-hr renewal, semi-static design.

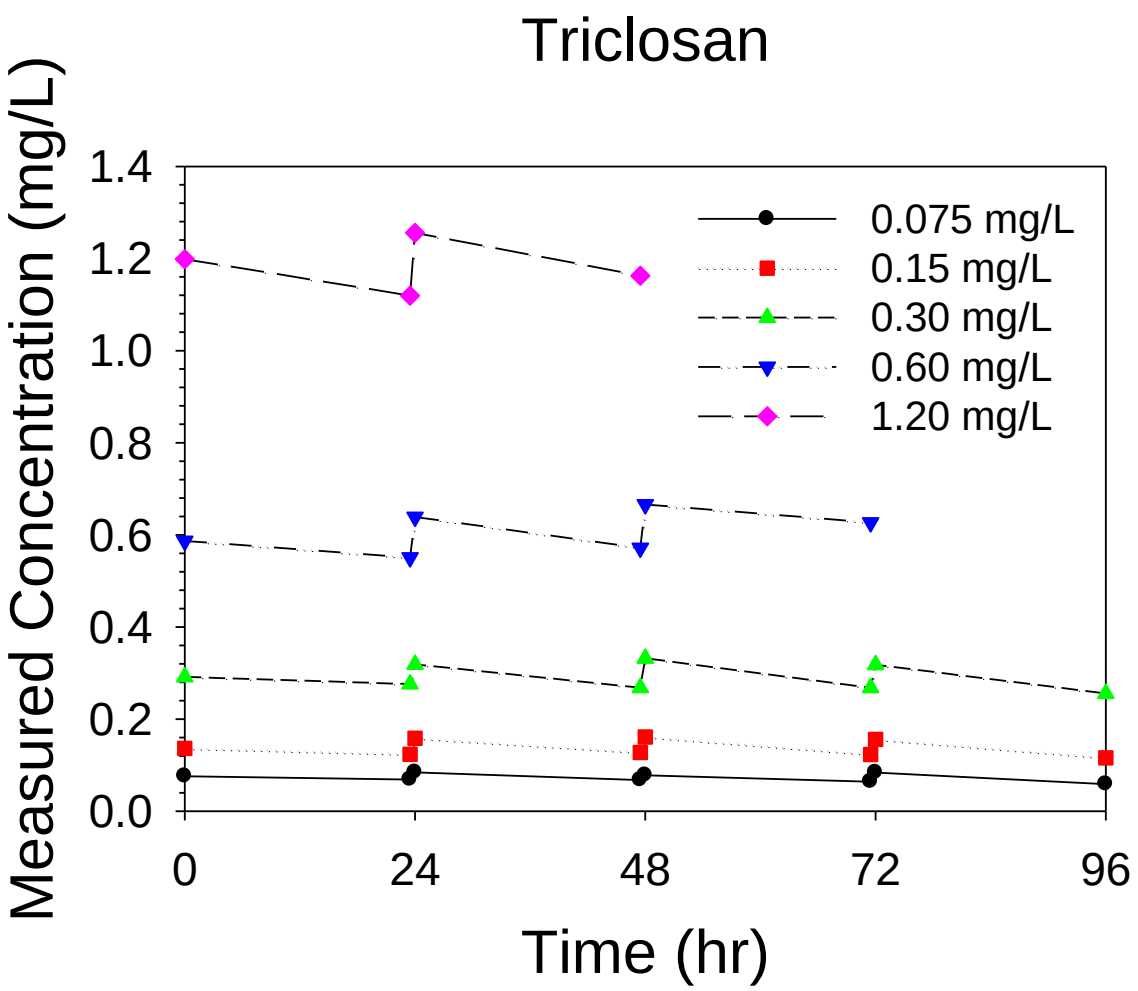

Figure 6. Pattern of measured triclosan concentrations in the FET using a 24-hr renewal, semi-static design.

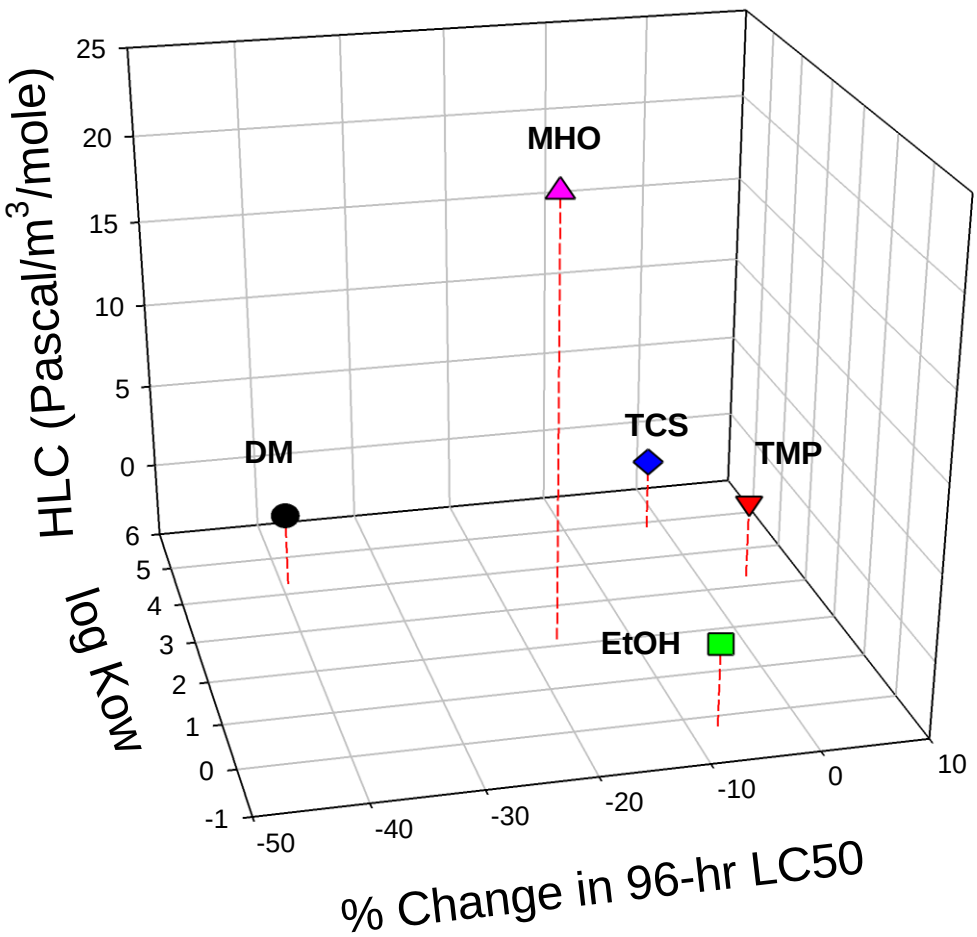

Figure 7. Relationship of physical chemical variables (HLC and Kow) to percent change in measured versus nominal 96-hr LC50s. DM and MHO indicate dibutyl maleate and 6-methyl-5-hepten-2-one, respectively.