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VALIDATION REPORT (PHASE 1) FOR THE ZEBRAFISH EMBRYO TOXICITY TEST PART 2

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VALIDATION REPORT (PHASE 1) FOR THE ZEBRAFISH EMBRYO TOXICITY TEST PART 2

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

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

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

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Validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test

Part II

Annexes VI, VII, VIII and IX

FOREWORD

This document presents **Part II** of the validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test (ZFET), on transferability, intra-, and inter-laboratory reproducibility for 7 chemicals. It includes Annexes VI, VII, VIII and IX. The main document and the first five annexes are included in **Part I** 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.

Annex VI - Statistical Report Phase 1b - Six Chemicals

Overview

This report refers to the statistical analysis as described in Annex 2 of the trial plan (TP_ZFET_OECD_1b_V01.1).

1. Methods

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

The primary model fit to the experimental results of this phase is the two-parameter logistic function. It has two parameters, LC_{50} and □, where

$$\Pr(Dead) = \frac{1}{1+\exp\left(\beta\left(x - LC_{50}\right)\right)}$$

Both x and LC_{50} are on the log-scale of concentration. This logistic regression model is one of the models recommended by the OECD Series on Testing and Assessment No. 54 for modelling quantal dose-response data[1]. Note that this model implies that there is no background mortality, and in fact observed background mortality does not contribute to model parameter estimation. Under this model, the control data role is solely to assess experimental quality.

If this model is an obviously poor fit to a given set of experimental data, the estimated LC50 and accompanying confidence intervals may be biased. In these cases, alternative models may be given consideration. For example, a three-parameter logistic model might fit better:

$$\Pr\left(Dead\right) = C + \frac{1-C}{1+\exp\left(\beta\left(x - LC_{50}\right)\right)}.$$

In this model the additional parameter C represents a positive non-zero background rate associated with the control group. This model is identical to the two-parameter logistic when C=0. While this equivalence is true and the model has the advantage of using the control data to estimate model parameters, there are drawbacks to using the three-parameter model exclusively. One is that when the background parameter is estimated to be very small or zero, the numerical calculations are sometimes unstable. More importantly, because we use at most 20 replicates per group, when background mortality is observed the only nonzero percentages possible are 1/20 = 5% and 2/20 = 10%, so there is a good chance that the parameter C will overestimate background mortality. Each of these is well above our historical experience, so the two models effectively trade bias in one direction for bias in another.

1.2. Quality criteria for fitting the model

Because these models must be fit using iterative numerical calculations, convergence of the numerical model fitting process must be confirmed prior to any other evaluation. Upon that confirmation, the fit of the primary model is checked using graphical summaries. If the model shows an obviously inappropriate fit, the estimated LC_{50} values may be biased, and alternative models will be investigated. It is preferable that all estimates and confidence intervals be based on a common model, so any secondary models will only be used if a strong justification exists.

[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

2.3. Confidence interval calculation

Confidence interval calculation is via the profile likelihood method[2]. In cases where the data provide adequate information for model estimation, profile likelihood confidence limits are nearly equivalent to the conventional intervals constructed from estimates and their standard errors. It has been shown that in very large samples they will become equivalent. Comparisons performed on data similar to those obtained in this phase demonstrate that the profile likelihood and conventional intervals are practically equivalent when the data are sufficiently informative, or well behaved, for fitting the model (results not shown). The advantage of profile likelihood intervals lies in cases where the data are not well behaved (described below), where, unlike the conventional intervals, then profile likelihood intervals will not be unrealistically wide, or narrow.

It is assumed that the tested concentrations reasonably bound the concentrations through which the response traverses the 0 to 100% response range. Because the embryos are tested in groups, a 'well-behaved' experimental result can be defined in terms of the number of informative concentration groups. Under the test design used for this validation, the informative groups will usually be the smallest concentration with partial mortality, the largest with partial mortality, plus all groups between, regardless of their mortality rates. More formally, in order to capture special cases, a group is informative if one of the following three conditions holds:

1. The group experiences partial toxicity, in the sense that at least one survival AND at least one death occurs (ie, the percentage of deaths is NOT 0 or 100%),
2. The group percentage is 0%, but at least one concentration BELOW the one under consideration is anything greater than 0%,
3. The group percentage is 100%, but at least one concentration ABOVE the one under consideration is anything less than 100%.

By these definitions informative groups must be consecutive in the concentration scale, and when two or more occur, that experimental result would be considered 'well-behaved'.

There are of course obvious cases for which an experimental result would be considered suboptimal. For example, if all of the response rates observed are less than 50%, or all greater than 50%, or all nearly equal to 50%, it should be obvious that these data will not provide good information on the LC_{50}. More commonly, the experimental results obtained do largely cover the full response range, but fewer than two informative groups occur. The two specific cases of concern are:

1. A single group experiences something between 0 and 100% mortality, and all groups at concentrations below it experience 0% mortality, and all groups at concentrations above it experience 100% (see 2.3.1 Case 1).
2. All groups experience only 0 or 100% mortality, and all of the 0% groups are in concentrations lower than all of the 100% groups (see 2.3.1 Case 2).

When the observed result is suboptimal, there is essentially no information for bounding the steepness of the concentration-response curve. The slope can be arbitrarily steep and, due to artefacts of the numerical computations, the conventional confidence intervals are either far too narrow, or too wide.[3] The profile

[2] Meeker, W.Q., Escobar, L.A. (1995): Teaching about approximate confidence regions based on maximum likelihood estimation. *The American Statistician*, v49, 48-53.

[3] Environment Canada (2005 with amendments from 2007): Guidance document on statistical methods for environmental toxicity tests/Method Development and Application Section. Section 4.

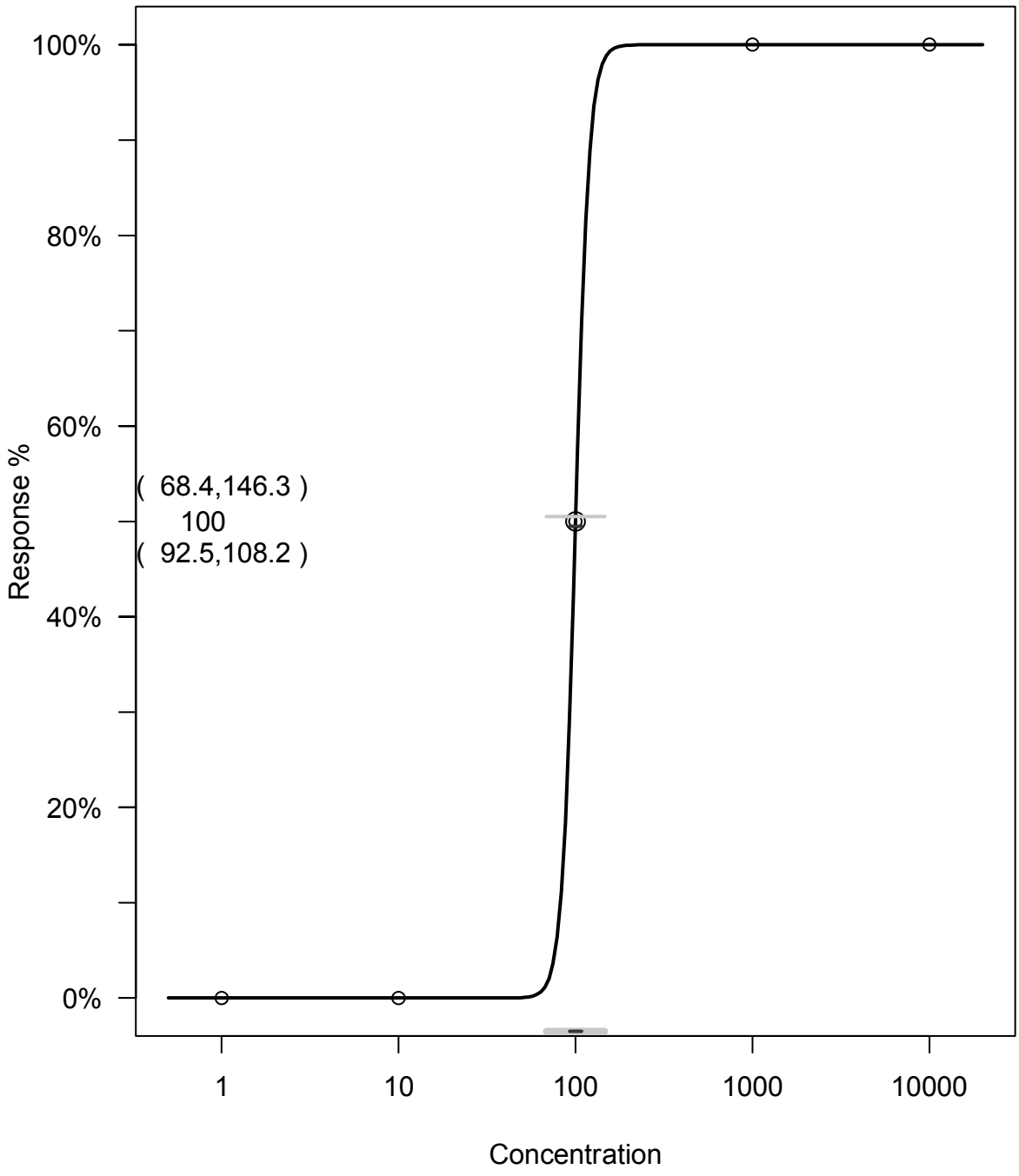
likelihood method for confidence interval construction is not susceptible to these problems. Profile likelihood intervals are more difficult to calculate (and hence the reason that the method is not more commonly implemented). A specialized program was developed to perform the calculations (available on request).

Other approaches were considered in the two suboptimal cases described above, such as the Spearman-Kärber or binomial method. However, this would result in different point estimates for the LC_{50} values for the within/between laboratories comparison (reliability) and therefore it was not implemented.

2.3.1. Illustrative Examples

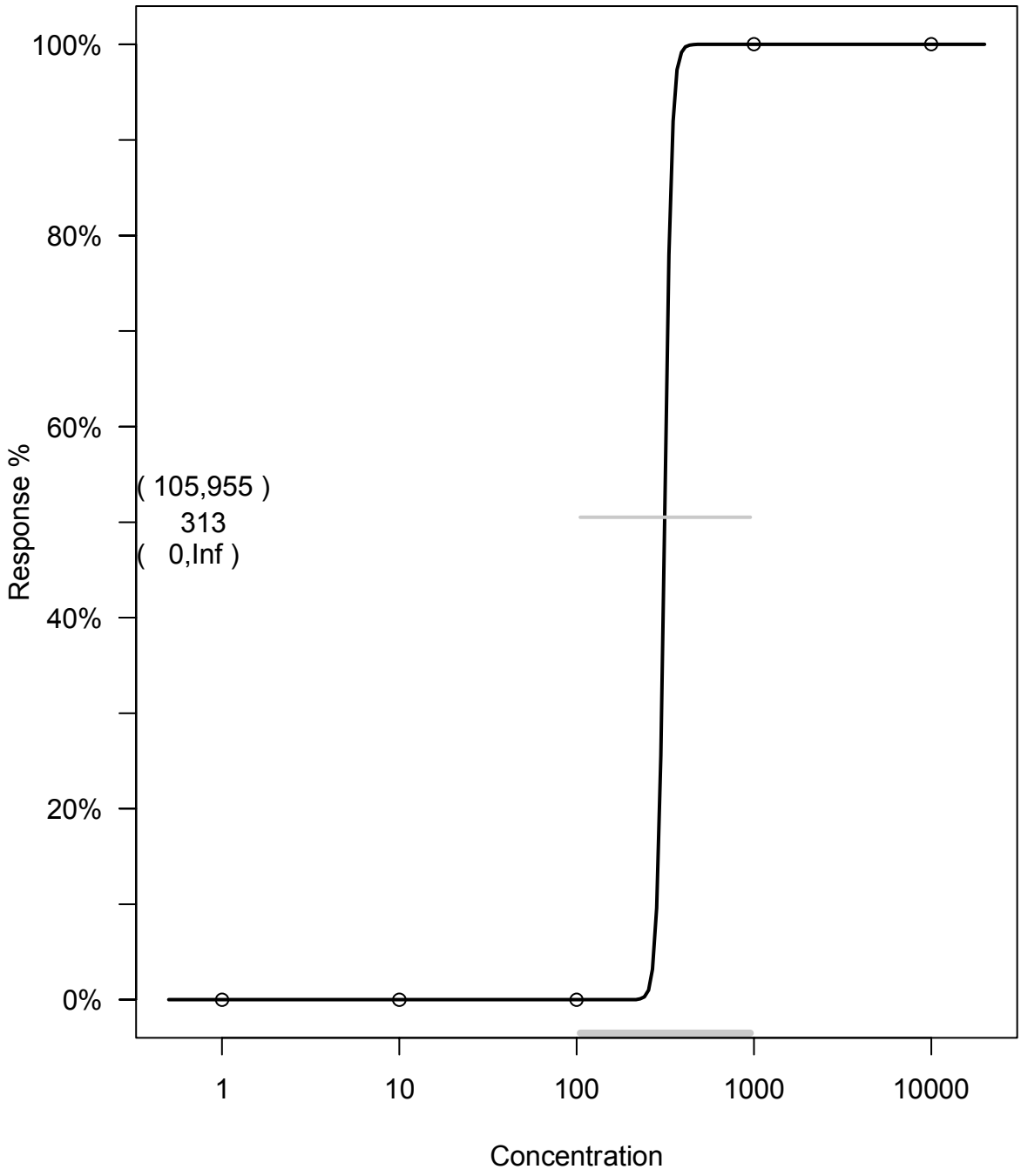
Case 1: Single partial response (one informative concentration)

The conventional interval (red, lower set of numbers) is very narrow around the concentration that has 50% response. It cannot be known that the LC_{50} lies in such a narrow window. The profile likelihood interval is much wider (blue, upper set of numbers).



Case 2: No informative concentrations (every group is either 0 or 100%)

In this case, the conventional interval does not even exist, because the estimated standard error of the LC_{50} is zero, so the interval is arbitrarily wide. The profile likelihood interval is intuitively correct: the LC_{50} is somewhere between the two concentrations that bracket the 50% response.



2.4. Calculations

All model-based calculations were performed with R version 2.12.0[4], using the standard numerical optimization functions available in the default installation of R. The profile likelihood calculations have been thoroughly tested, and were also found to be in agreement with profile likelihood estimation in SAS/STAT LOGISTIC procedure[5]. Statistical tests on the control data were performed in StatXact v4[6].

2.5. Internal control analysis

The potential effects of a 'halo effect' of the toxicity due to treatment in neighbouring wells will be assessed by stratified Cochran-Armitage tests of trend in proportions. The data from internal controls will be summarized at various levels (the strata), where the statistical analysis will attempt to detect that more control deaths occur in plates on which high toxicity is experienced in the neighbouring test article wells, compared to controls tested in plates with low toxicity in the test article wells. Alternatively, a correlation analysis can be conducted in which, rather than the actual exposure concentration, the toxicity of the test article is used as the predictor for control well toxicity.

2.6 Summarization of results

This phase of the validation is focused on reproducibility, although it is also possible to evaluate the ability of the ZFET to distinguish these chemicals of widely separated toxicity levels. The estimated LC_{50} values and profile likelihood confidence intervals for the six test articles (sodium chloride, ethanol, dibutyl maleate, 2,3,6-trimethylphenol, 5-methyl-6-hepten-2-one, and triclosan) are calculated for each qualified run at 48 and 96h. This information is further summarized graphically and statistically (see Appendix A).

[4] R Development Core Team (2010). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

[5] SAS Institute Inc. 2010. SAS/STAT® 9.22 User's Guide. Cary, NC: SAS Institute Inc.

[6] Cytel Software Corp. 1998. StatXact 4 For Windows: Statistical Software for Exact Nonparametric Inference. Cambridge, MA: CYTEL Software Corp.

3. Results

The designated primary model

$$\Pr(Dead) = \frac{1}{1+\exp\left(\beta\left(x - LC_{50}\right)\right)}$$

is adequate for all models fit to data from this phase. None of the model fits is sufficiently improved by alternative models to justify this added complexity to the analysis and interpretation.

3.1 Run-level summaries

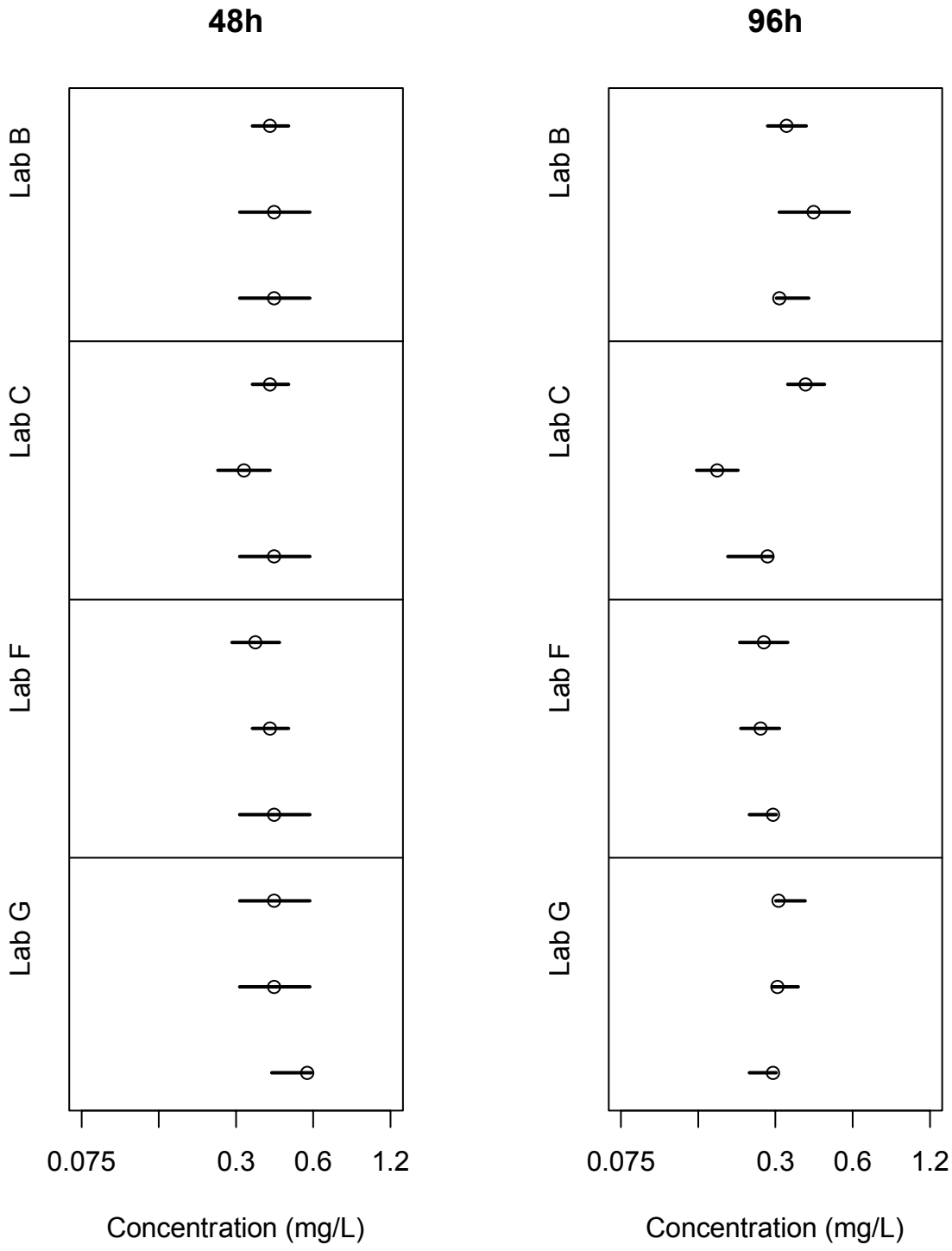
3.1.1. Triclosan

Table 1: LC_{50} values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Triclosan

		48h (mg/L)				96h (mg/L)			
Lab	Run	LC_{50}	Lower	Upper	Inform	LC_{50}	Lower	Upper	Inform
B	1	0.407	0.348	0.480	3	0.332	0.396	0.279	3
	2	0.423	0.310	0.581	0	0.423	0.581	0.310	0
	3	0.423	0.310	0.581	0	0.311	0.404	0.302	1
	Mean	0.418				0.355			
C	1	0.407	0.348	0.480	3	0.393	0.465	0.335	3
	2	0.322	0.255	0.407	3	0.178	0.214	0.148	2
	3	0.423	0.310	0.581	0	0.279	0.292	0.196	1
	Mean	0.384				0.283			
F	1	0.357	0.289	0.443	3	0.270	0.335	0.217	3
	2	0.407	0.348	0.480	3	0.263	0.311	0.220	3
	3	0.423	0.310	0.581	0	0.294	0.302	0.237	1
	Mean	0.396				0.275			
G	1	0.423	0.310	0.581	0	0.309	0.391	0.301	1
	2	0.423	0.310	0.581	0	0.305	0.368	0.291	1
	3	0.568	0.414	0.589	1	0.294	0.302	0.237	1
	Mean	0.471				0.302			
	Grand Mean	0.417				0.304			

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix A)

Figure 1: LC_{50} values and 95% confidence limits for tests of Triclosan in the Zebrafish Embryo Toxicity Test



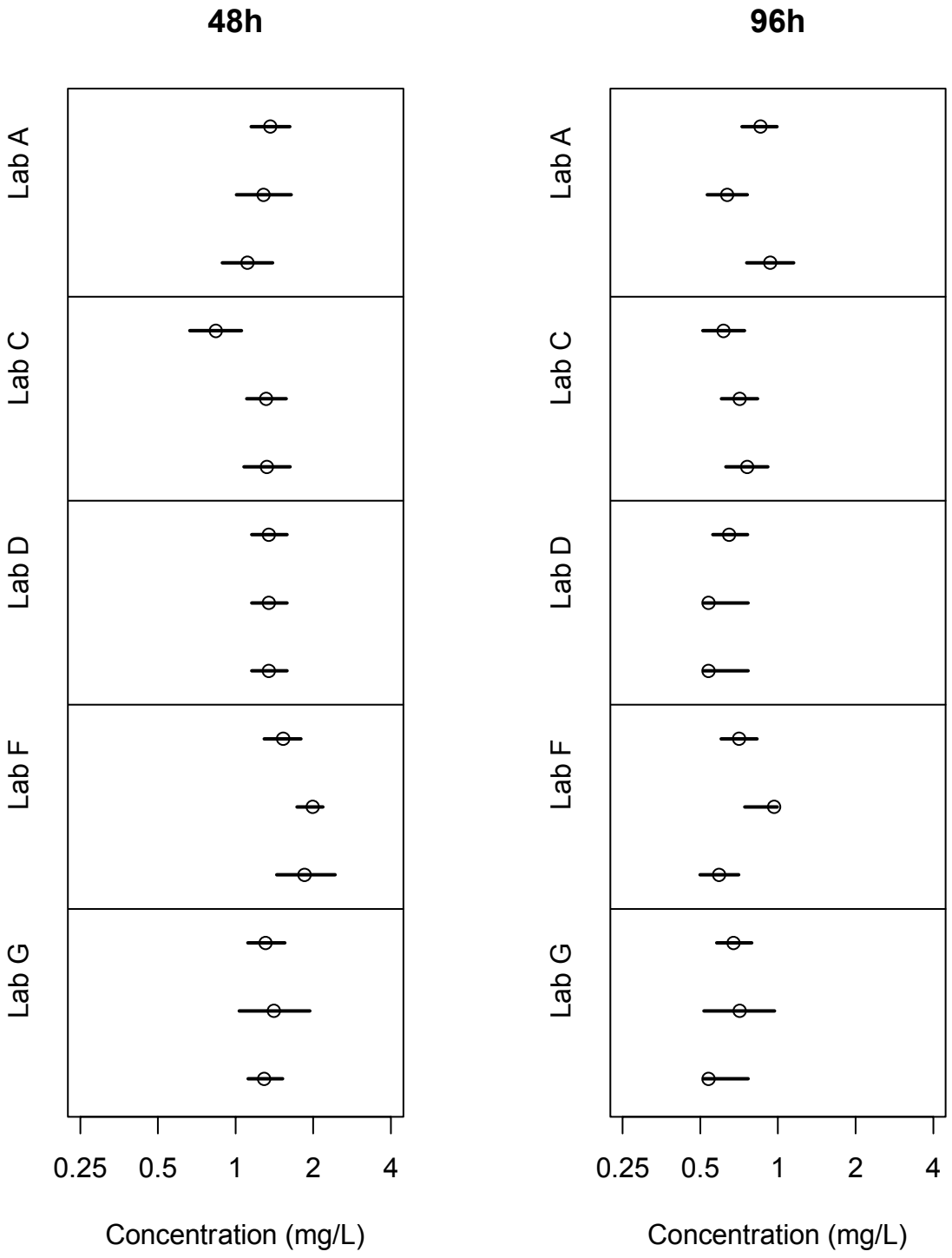
3.1.2. Dibutyl Maleate

Table 2: LC_{50} values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Dibutyl Maleate

		48h (mg/L)				96h (mg/L)			
Lab	Run	LC_{50}	Lower	Upper	Inform	LC_{50}	Lower	Upper	Inform
A	1	1.360	1.150	1.62	3	0.855	0.990	0.725	2
	2	1.280	1.010	1.64	4	0.635	0.761	0.531	3
	3	1.110	0.888	1.39	4	0.933	1.150	0.757	3
	Mean	1.250				0.807			
C	1	0.838	0.664	1.05	3	0.615	0.742	0.512	3
	2	1.320	1.110	1.57	3	0.709	0.833	0.604	2
	3	1.320	1.080	1.63	4	0.758	0.913	0.628	3
	Mean	1.160				0.694			
D	1	1.340	1.160	1.58	2	0.646	0.761	0.559	2
	2	1.340	1.160	1.58	2	0.538	0.766	0.514	1
	3	1.340	1.160	1.58	2	0.538	0.766	0.514	1
	Mean	1.340				0.574			
F	1	1.530	1.290	1.79	3	0.707	0.830	0.603	2
	2	1.990	1.730	2.18	1	0.965	0.992	0.742	1
	3	1.850	1.450	2.43	4	0.591	0.704	0.499	2
	Mean	1.790				0.754			
G	1	1.310	1.120	1.55	3	0.672	0.790	0.578	2
	2	1.410	1.030	1.94	0	0.709	0.969	0.516	0
	3	1.290	1.120	1.52	2	0.538	0.766	0.514	1
	Mean	1.340				0.640			
	Grand Mean	1.380				0.694			

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix A)

Figure 2: LC_{50} values and 95% confidence limits for tests of Dibutyl Maleate in the Zebrafish Embryo Toxicity Test



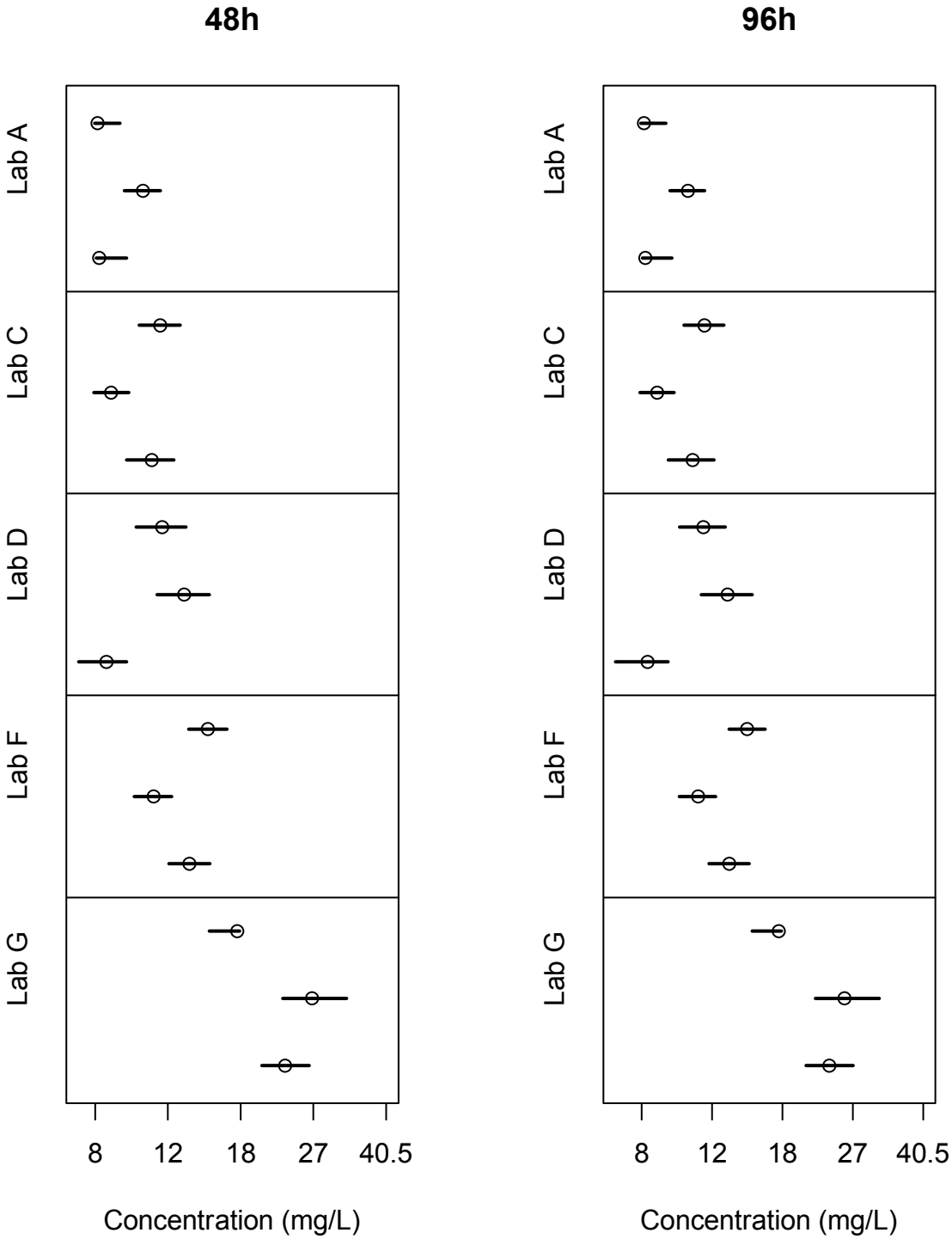
3.1.3 2,3,6-Trimethylphenol

Table 3: LC_{50} values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with 2,3,6-Trimethylphenol

		48h (mg/L)				96h (mg/L)			
Lab	Run	LC_{50}	Lower	Upper	Inform	LC_{50}	Lower	Upper	Inform
A	1	8.11	7.97	9.19	1	8.11	9.19	7.97	1
	2	10.40	9.41	11.50	2	10.40	11.50	9.41	2
	3	8.17	8.05	9.53	1	8.17	9.53	8.05	1
	Mean	8.91				8.91			
C	1	11.50	10.20	12.90	2	11.50	12.90	10.20	2
	2	8.75	7.94	9.65	2	8.75	9.65	7.94	2
	3	11.00	9.53	12.40	3	10.70	12.10	9.33	3
	Mean	10.40				10.30			
D	1	11.60	10.10	13.30	3	11.40	13.00	9.96	3
	2	13.10	11.30	15.10	3	13.10	15.10	11.30	3
	3	8.52	7.29	9.53	2	8.29	9.32	6.89	2
	Mean	11.10				11.00			
F	1	15.00	13.50	16.70	2	14.70	16.30	13.20	2
	2	11.10	9.95	12.30	3	11.10	12.30	9.95	3
	3	13.50	12.10	15.10	2	13.20	14.90	11.80	2
	Mean	13.20				13.00			
G	1	17.60	15.10	17.90	1	17.60	17.90	15.10	1
	2	26.80	22.80	32.50	4	25.80	31.50	21.80	4
	3	23.10	20.20	26.40	4	23.60	27.00	20.60	4
	Mean	22.50				22.30			
	Grand Mean	13.20				13.10			

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix A)

Figure 3: LC_{50} values and 95% confidence limits for tests of 2,3,6-Trimethylphenol in the Zebrafish Embryo Toxicity Test



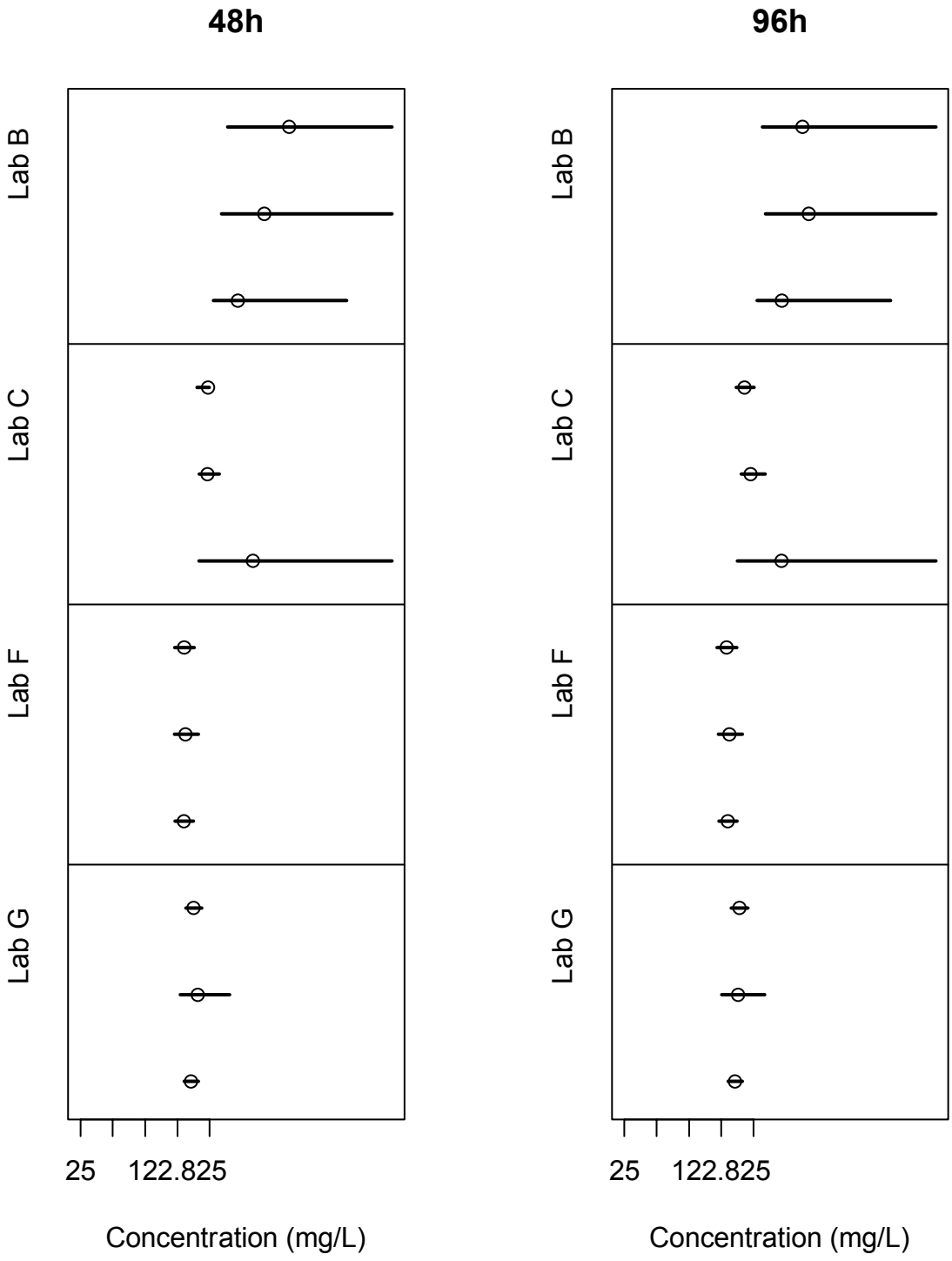
3.1.4. 6-Methyl-5-hepten-2-one

Table 4: LC50 values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with 6-Methyl-5-hepten-2-one

		48h (mg/L)				96h (mg/L)			
Lab	Run	LC_{50}	Lower	Upper	Inform	LC_{50}	Lower	Upper	Inform
B	1	773*	282	4180	5	465*	4180	241	5
	2	514*	253	4180	4	516*	4180	254	4
	3	332*	222	1980	4	332*	1980	222	4
	Mean	539*				438*			
C	1	203	170	207	1	180	210	157	3
	2	201	176	245	2	199	253	171	2
	3	426*	176	4180	5	330*	4180	160	5
	Mean	277*				236*			
F	1	137	118	162	3	134	158	115	3
	2	140	117	173	5	140	173	117	5
	3	137	119	160	3	137	160	119	3
	Mean	138				137			
G	1	160	141	183	3	165	190	144	3
	2	172	129	290	5	161	251	124	5
	3	154	137	174	2	154	174	137	2
	Mean	162				160			
	Grand Mean	279*				243*			

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix A)
* The estimated LC50 value is higher than the highest test concentration (208.02 mg/L)

Figure 4: LC50 values and 95% confidence limits for tests of 6-Methyl-5-hepten-2-one in the Zebrafish Embryo Toxicity Test



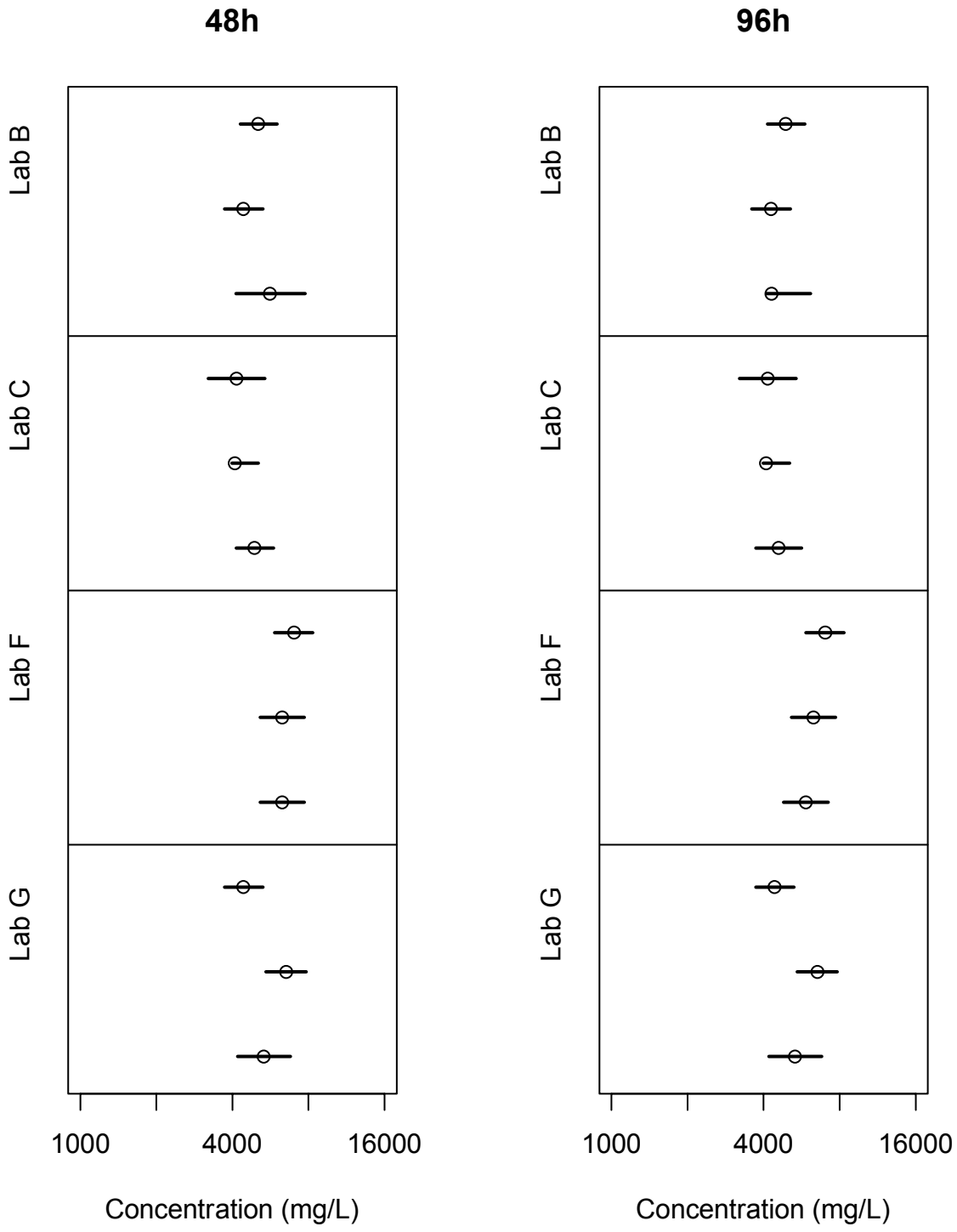
3.1.5. Sodium Chloride

Table 5: LC_{50} values and confidence intervals of the Zebrafish Embryo Toxicity Test – three runs with Sodium Chloride

		48h (mg/L)				96h (mg/L)			
Lab	Run	LC_{50}	Lower	Upper	Inform	LC_{50}	Lower	Upper	Inform
B	1	5060	4300	6000	3	4890	5820	4140	3
	2	4420	3720	5280	3	4280	5110	3590	3
	3	5640	4130	7750	0	4300	6130	4110	1
	Mean	5040				4490			
C	1	4150	3200	5370	4	4150	5370	3200	4
	2	4090	3980	5060	1	4090	5060	3980	1
	3	4890	4140	5820	2	4590	5650	3730	3
	Mean	4370				4270			
F	1	7010	5870	8320	3	7010	8320	5870	3
	2	6300	5160	7710	4	6300	7710	5160	4
	3	6300	5160	7710	4	5870	7200	4800	4
	Mean	6530				6390			
G	1	4420	3720	5280	3	4420	5280	3720	3
	2	6530	5430	7820	4	6530	7820	5430	4
	3	5320	4190	6790	4	5320	6790	4190	4
	Mean	5420				5420			
	Grand Mean	5340				5140			

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix A)

Figure 5: LC_{50} values and 95% confidence limits for tests of Sodium Chloride in the Zebrafish Embryo Toxicity Test



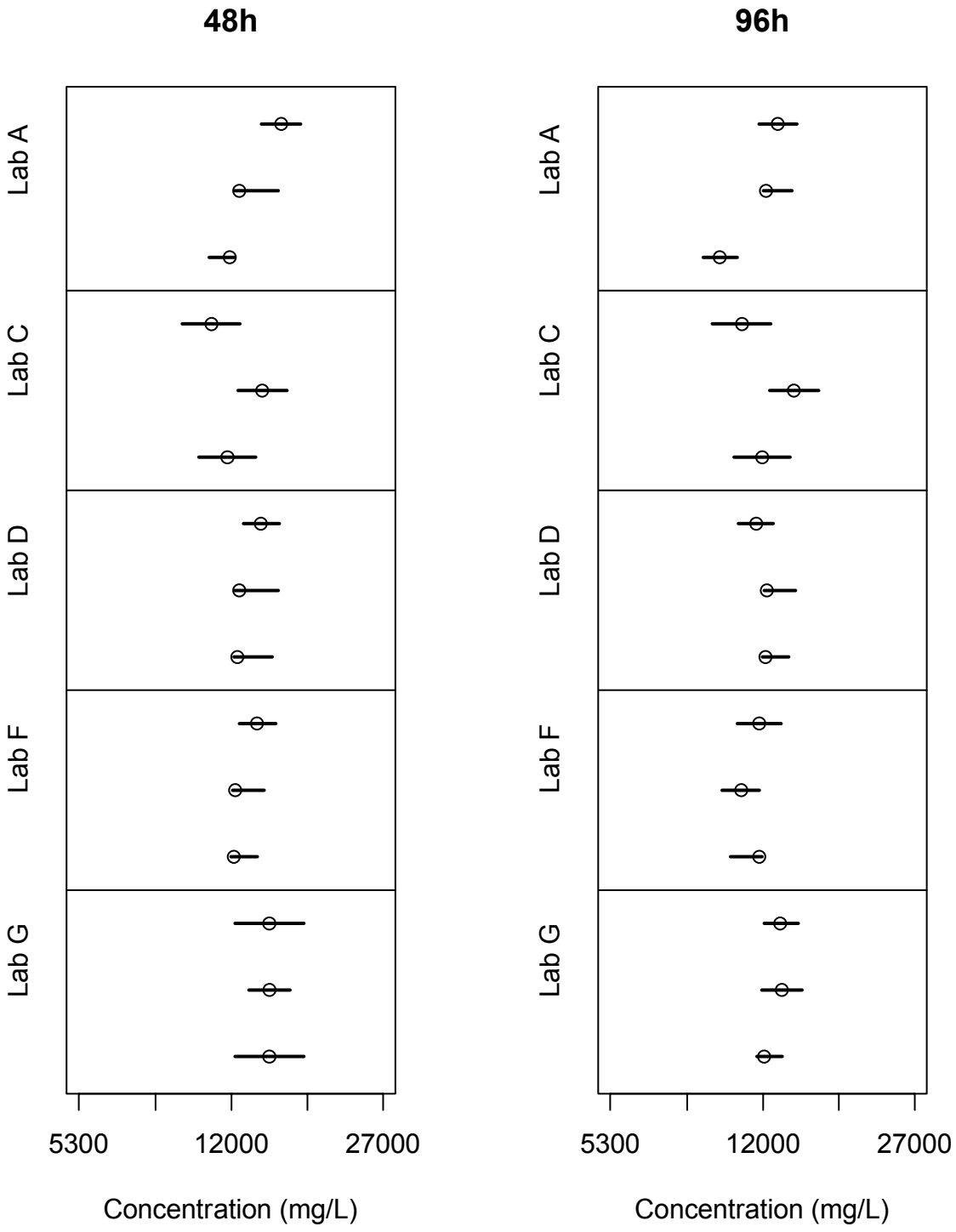
3.1.6. Ethanol

Table 6: LC_{50} and confidence intervals (mg/L) of the Zebrafish Embryo Toxicity Test – three runs with Ethanol

		48h (mg/L)				96h (mg/L)			
Lab	Run	LC_{50}	Lower	Upper	Inform	LC_{50}	Lower	Upper	Inform
A	1	15700	14100	17400	4	13000	14400	11700	3
	2	12500	12200	15400	1	12200	14000	12000	1
	3	11900	10600	12200	1	9510	10500	8710	2
	Mean	13400				11600			
C	1	10800	9220	12600	4	10700	12500	9140	4
	2	14100	12400	16100	4	14100	16100	12400	4
	3	11700	10100	13700	3	11900	13900	10300	3
	Mean	12200				12300			
D	1	14000	12800	15500	3	11600	12700	10500	2
	2	12500	12200	15400	1	12300	14300	12100	1
	3	12400	12200	14900	1	12200	13800	12000	1
	Mean	13000				12000			
F	1	13800	12500	15200	3	11800	13200	10500	3
	2	12300	12100	14300	1	10700	11800	9640	3
	3	12200	12000	13800	1	11800	11900	10100	1
	Mean	12700				11400			
G	1	14700	12200	17700	0	13200	14500	12100	2
	2	14700	13200	16400	4	13300	14800	11900	3
	3	14700	12200	17700	0	12100	13300	11600	1
	Mean	14700				12800			
	Grand Mean	13200				12000			

Inform = the number of informative concentrations (defined in Section 2.3; see figures in the Appendix A)

Figure 6: LC_{50} values and 95% confidence limits for tests of Ethanol in the Zebrafish Embryo Toxicity Test



3.2. Global Comparisons

An overall graphical summary of results appears in Figures 7 and 8. Chemicals are ordered by the average toxicity value estimated in this validation study. The gray shading within each chemical identifies the range of concentrations tested, where the vertical lines within each shaded region, along with the outer vertical edges, are the five concentration levels tested. The horizontal dashed lines within the gray-shaded regions separate the sets of three runs contributed by each participating lab. Letter codes for the labs are placed in the margins.

There is no overlap of estimated LC50s among these chemicals, and with the exception of obviously problematic runs of 6-Methyl-5-hepten-2-one, the confidence intervals also do not overlap. These problem cases are exclusively those for which the test failed to observe a concentration with mortality exceeding 50%. The estimated LC50s for each chemical fall within a fairly small range, with 2,3,6-Trimethylphenol being the large range of the well-behaved experimental results: about one-half order of magnitude range.

Table 7: LC50 ranges and range ratios

Chemical	Time (h)	Min LC_{50}	Max LC_{50}	Ratio
Triclosan	48	0.322	0.568	1.76
	96	0.178	0.423	2.38
Dibutyl Maleate	48	0.838	1.99	2.37
	96	0.538	0.965	1.79
2,3,6-Trimethylphenol	48	8.11	26.8	3.30
	96	8.11	25.8	3.18
6-Methyl-5-hepten-2-one	48	137	773	5.65
	96	134	516	3.85
Sodium Chloride	48	4090	7010	1.71
	96	4090	7010	1.71
Ethanol	48	10800	15700	1.45
	96	9510	14100	1.49

Figure 7: Global Summary of 48h test results

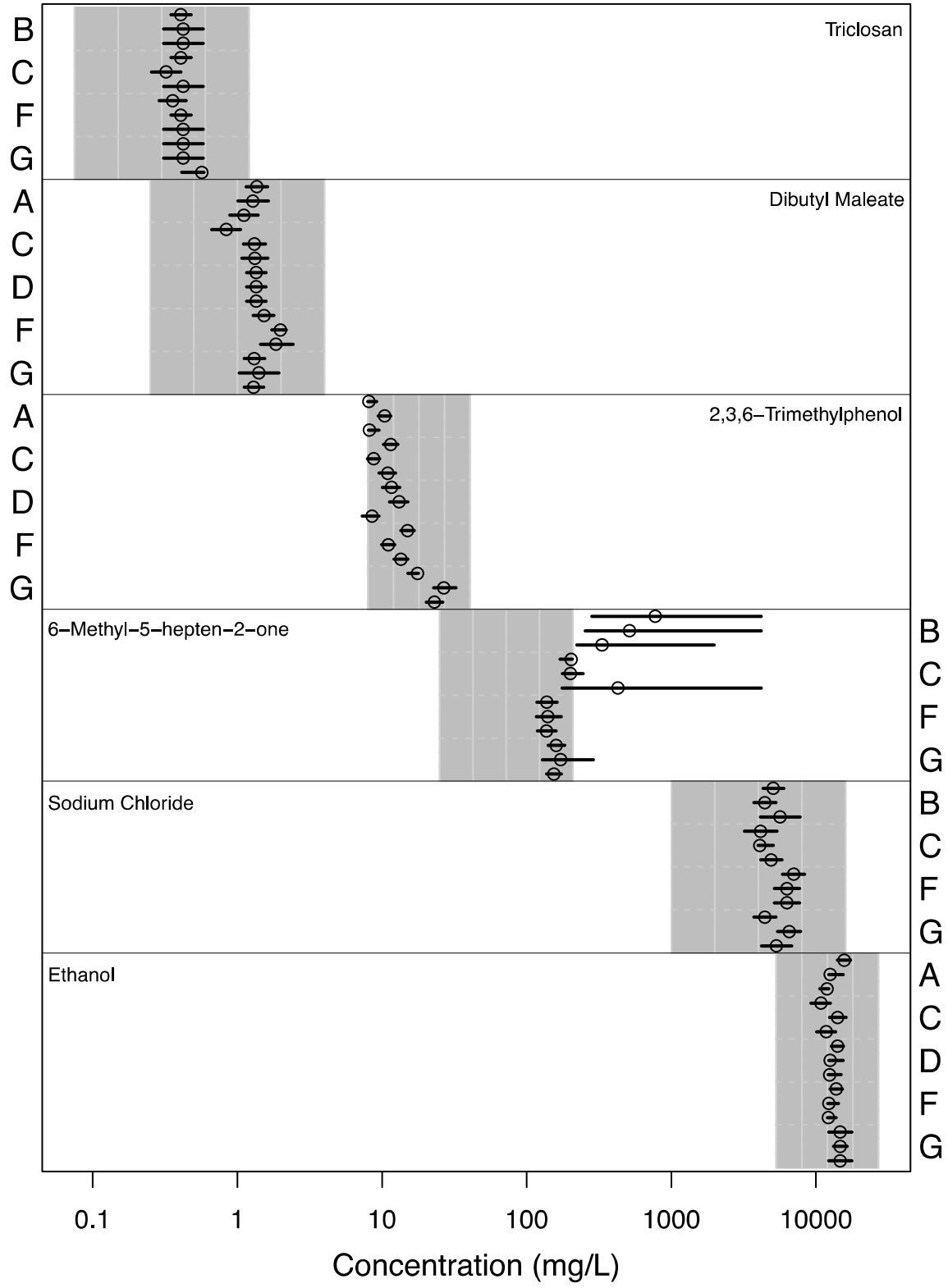
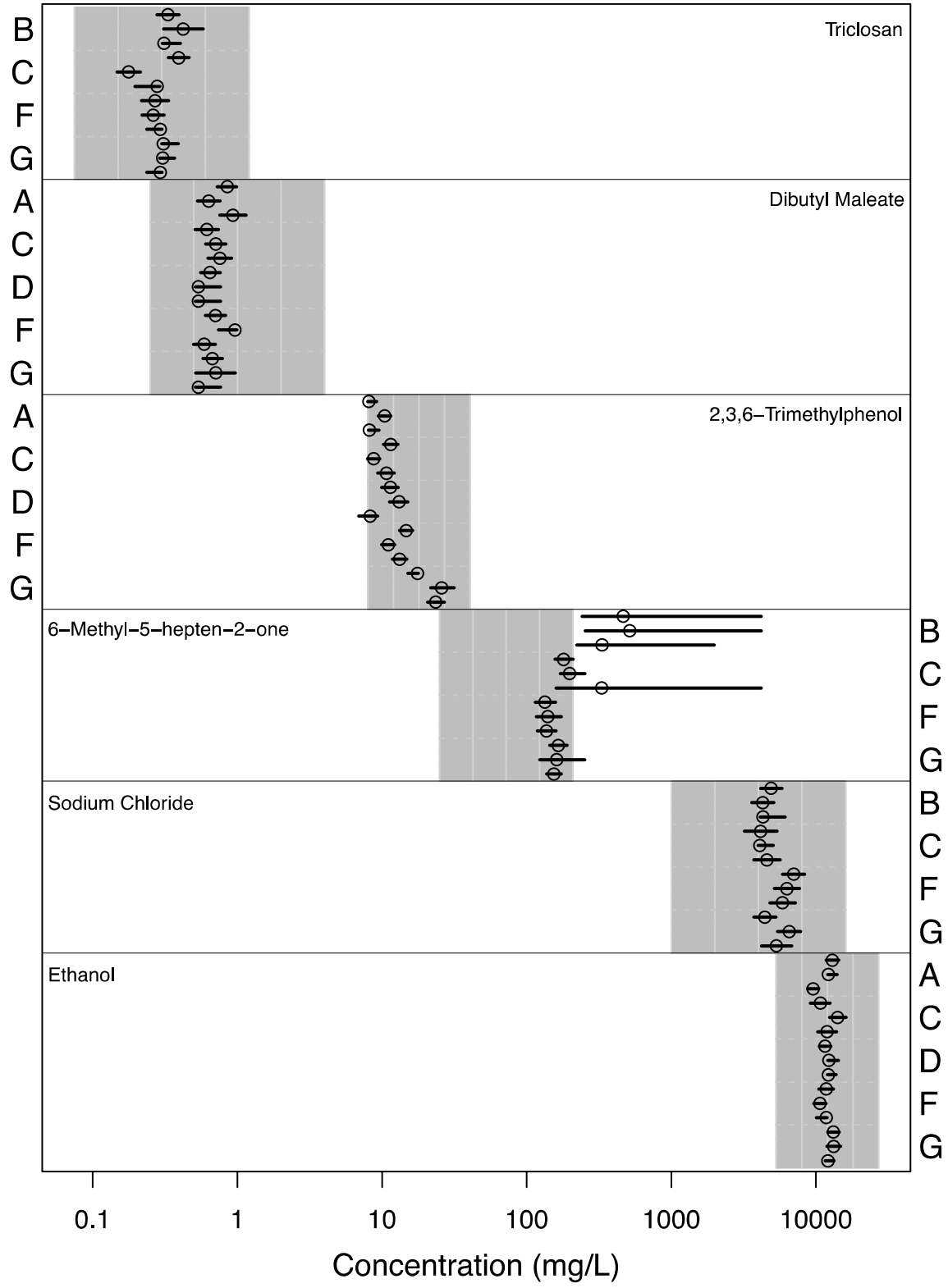


Figure 8: Global Summary of 96h test results



3.3. Intralaboratory variability

The calculated coefficients of variation per laboratory and test item are given in table 8.

Table 8: Intra-laboratory reproducibility - coefficients of variation (%) for 6 test items – three runs (--- indicates chemical not tested)

		Laboratory					
Time (h)	Chemical	A	B	C	D	F	G
48	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
96	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

3.3. Inter-laboratory variability

The inter-laboratory coefficients of variation were calculated based on the combined LC50 calculations and are given in Table 9.

Table 9: Inter-laboratory reproducibility - coefficients of variation for 6 test items based on means of three runs/lab

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

4. Effect on internal controls due to test article toxicity in neighbouring wells

Figures 9, 10, and 11 evaluate the correlation of treatment toxicity with internal control mortality. Because internal controls are paired with exactly one chemical, concentration, and time, there is a possibility that internal controls could respond to toxic effects due to treatments applied to neighbouring wells. The premise is that, particularly for volatile substances or highly toxic exposures, there may be a toxic effect on neighbouring control wells, even though no toxic treatment is directly applied.

In these figures, internal control mortality is the percentage calculated by pooling all data across labs and runs for a given chemical, concentration, and time, compared to a similar pooling of test article data on all plates tested with the identical chemical, concentration, and time. In total there are 74 data points: for each time point there are six chemicals and for each chemical there are six treatments (5 test article concentrations, 1 positive control) which accounts for 6x6 = 36 data points per time, plus the single ethanol control group in Triclosan for a total of 37 per time, or 74 total.

Individually, there are three chemicals with a positive correlation, and three with a negative correlation, though none are statistically significant. The 48 and 96h data are highly correlated in time (i.e. are nearly the same).

All of the data in Figures 9 and 10 are combined into Figure 11, where the colours and symbol styles of Figures 9 and 10 are carried over. A linear regression model is superimposed showing little or no effect of test article toxicity on matched internal control toxicity. In fact, the slope ($\hat{\beta}_{LR} = -0.0008$) is slightly negative, and nowhere close to statistically significant (p=0.9).

These data are further broken down by chemical and time in Figures 10 and 11. Nothing noteworthy appears in these displays.

Figure 9: 48h Correlation of internal control mortality as function of test article toxicity, for each chemical separately

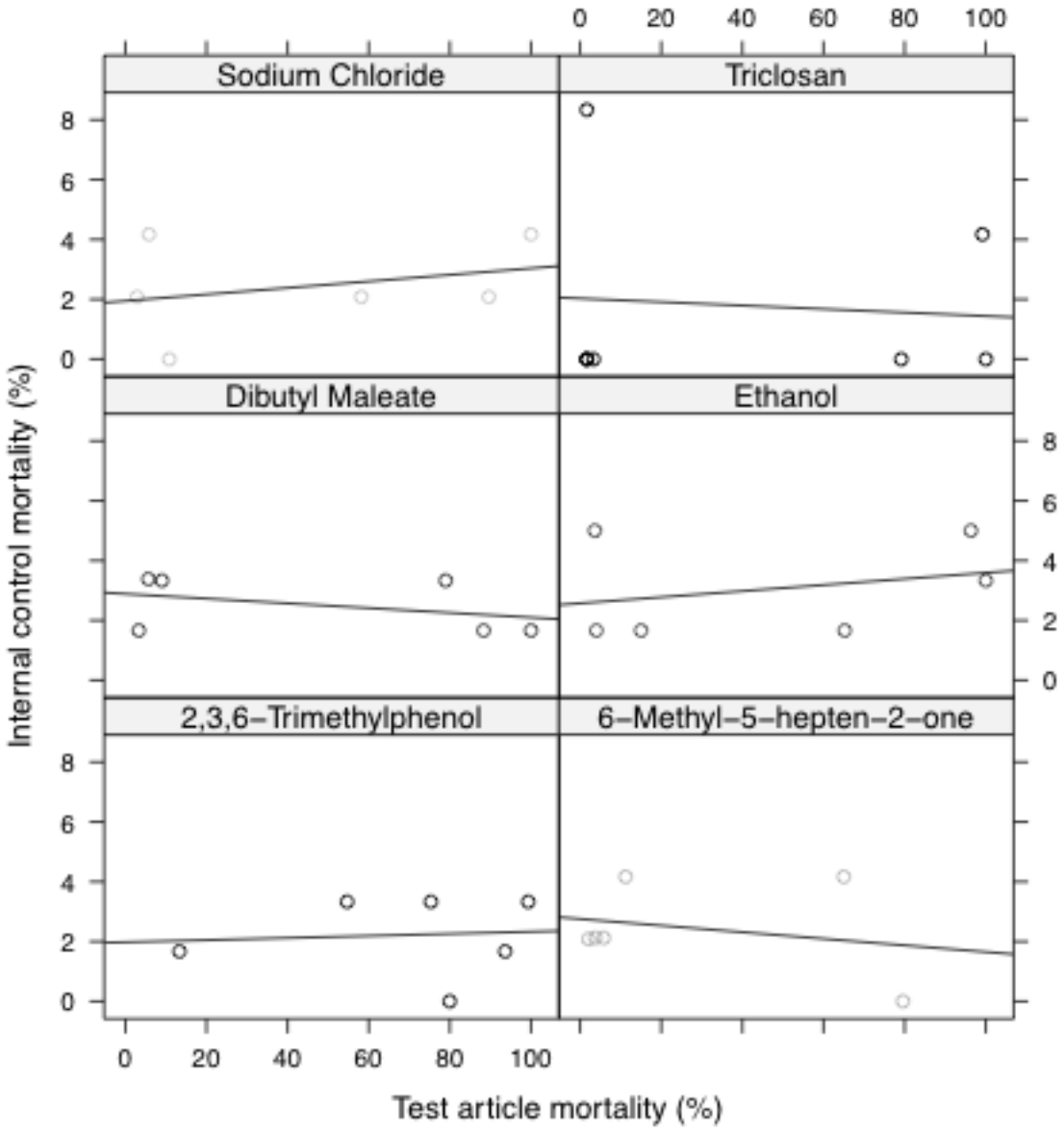


Figure 10: 96h Correlation of internal control mortality as function of test article toxicity, for each chemical separately

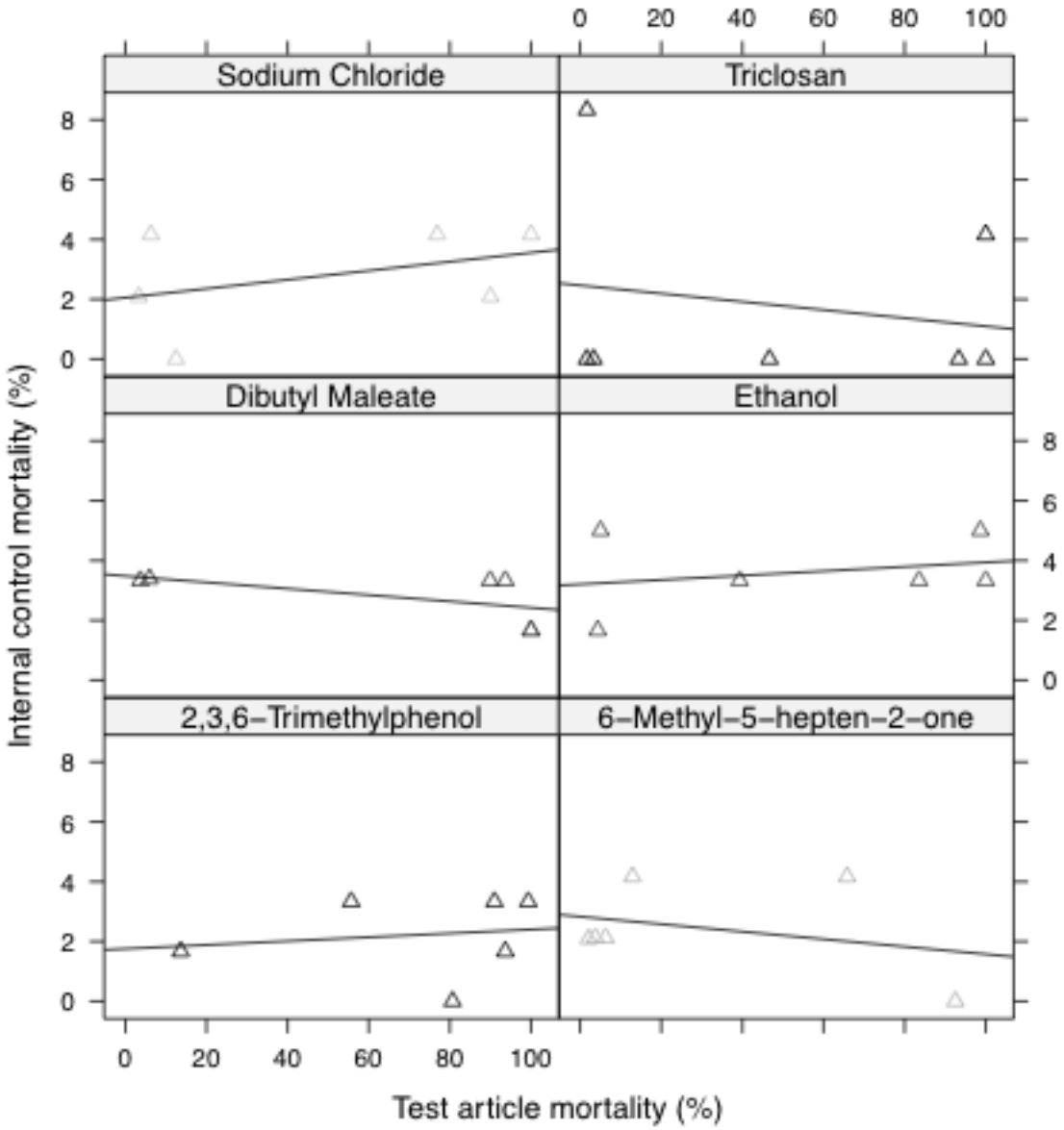
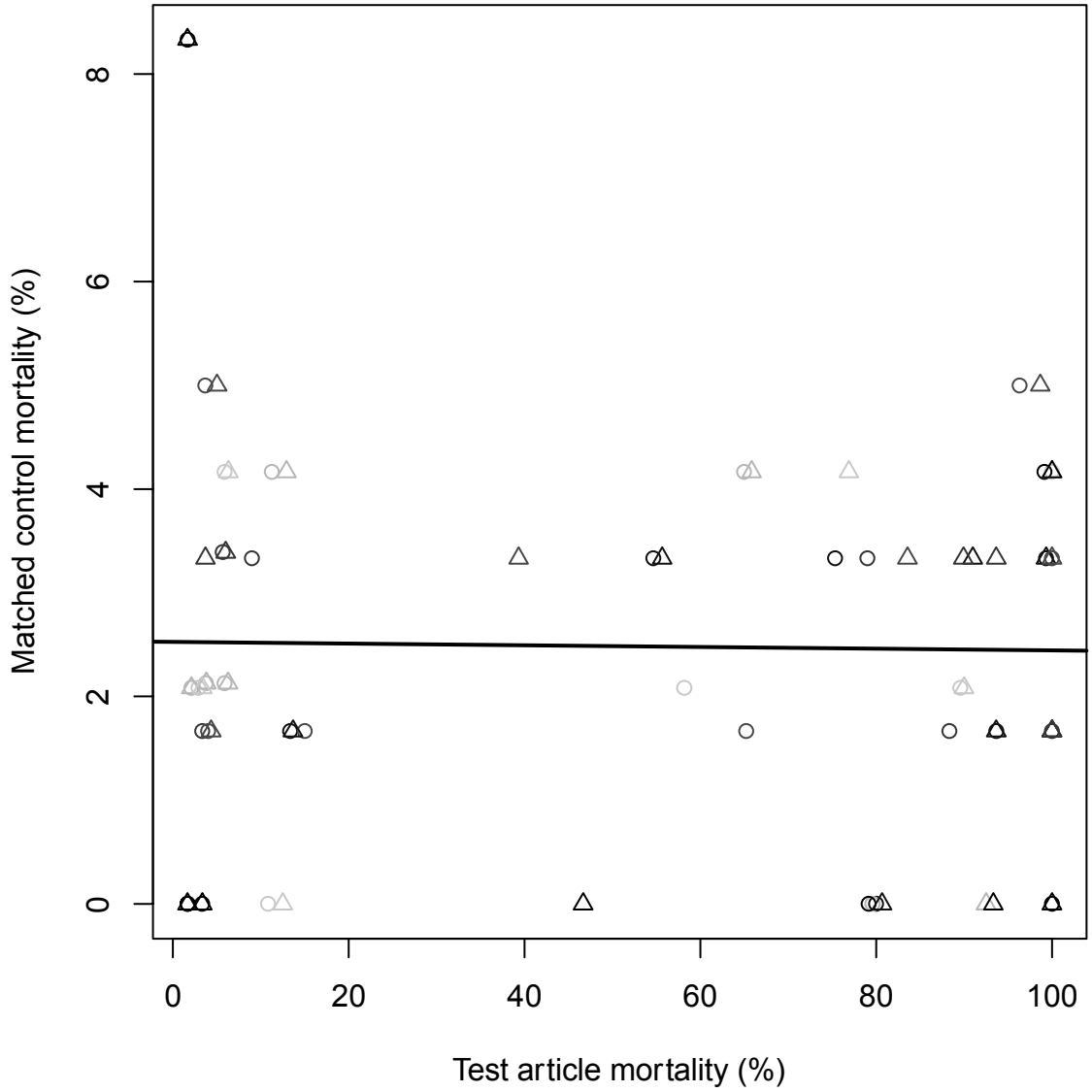


Figure 11: Correlation of internal control mortality as function of test article toxicity



Overview internal, external and positive controls per laboratory

The most remarkable finding on the external controls is the laboratory variability in detection of control deaths. Lab C in particular tends to be high for both internal and external control observations, but is most remarkable for its internal controls experiencing mortality at twice the rate of its external controls.

Table 10.1: Summarised overview: external controls

Time (h)	Lab	Dead	Total	Percent	Runs
48	A	7	216	3.24	9
	B	2	216	0.93	9
	C	9	432	2.08	18
	D	0	212	0.00	9
	F	8	432	1.85	18
	G	10	432	2.31	18
	Total	36	1940	1.86	81
96	A	7	216	3.24	9
	B	2	214	0.93	9
	C	10	431	2.32	18
	D	0	216	0.00	9
	F	10	432	2.31	18
	G	10	432	2.31	18
	Total	39	1941	2.01	81

Table 10.2: Summarised overview: internal controls

Time (h)	Lab	Dead	Total	Percent	Runs
48	A	7	216	3.24	9
	B	1	226	0.44	9
	C	19	443	4.29	18
	D	3	216	1.39	9
	F	9	444	2.03	18
	G	9	444	2.03	18
	Total	48	1989	2.41	81
96	A	7	216	3.24	9
	B	1	226	0.44	9
	C	20	443	4.51	18
	D	3	216	1.39	9
	F	11	444	2.48	18
	G	10	444	2.25	18
	Total	52	1989	2.61	81

Table 11: Summarised overview external solvent controls for Triclosan because different solvent has been used (0.1% Ethanol in dilution water)

Time (h)	Lab	Dead	Total	Percent	Runs
48	B	1	60	1.67	3
	C	1	60	1.67	3
	F	1	60	1.67	3
	G	1	60	1.67	3
	Total	4	240	1.67	12
96	B	1	60	1.67	3
	C	1	60	1.67	3
	F	1	60	1.67	3
	G	1	60	1.67	3
	Total	4	240	1.67	12

Table 12: Summarised overview positive controls

Time (h)	Lab	Dead	Total	Percent	Runs
48	A	128	180	71.11	9
	B	170	180	94.44	9
	C	316	358	88.27	18
	D	154	180	85.56	9
	F	171	360	47.50	18
	G	239	360	66.39	18
	Total	1178	1618	72.81	81
96	A	163	180	90.56	9
	B	172	180	95.56	9
	C	321	356	90.17	18
	D	179	180	99.44	9
	F	268	360	74.44	18
	G	329	360	91.39	18
	Total	1432	1616	88.61	81

5. Comments

The level of replication (number of laboratories assessing a given compound) in this phase is relatively small but typical of many validation efforts. This limits the statistical summarization to some degree. It is difficult to separate the potential sources of variability in these data (run-to-run variability within lab and chemical, and lab-to-lab within chemical), particularly if they vary as a function of lab, chemical tested, etc.

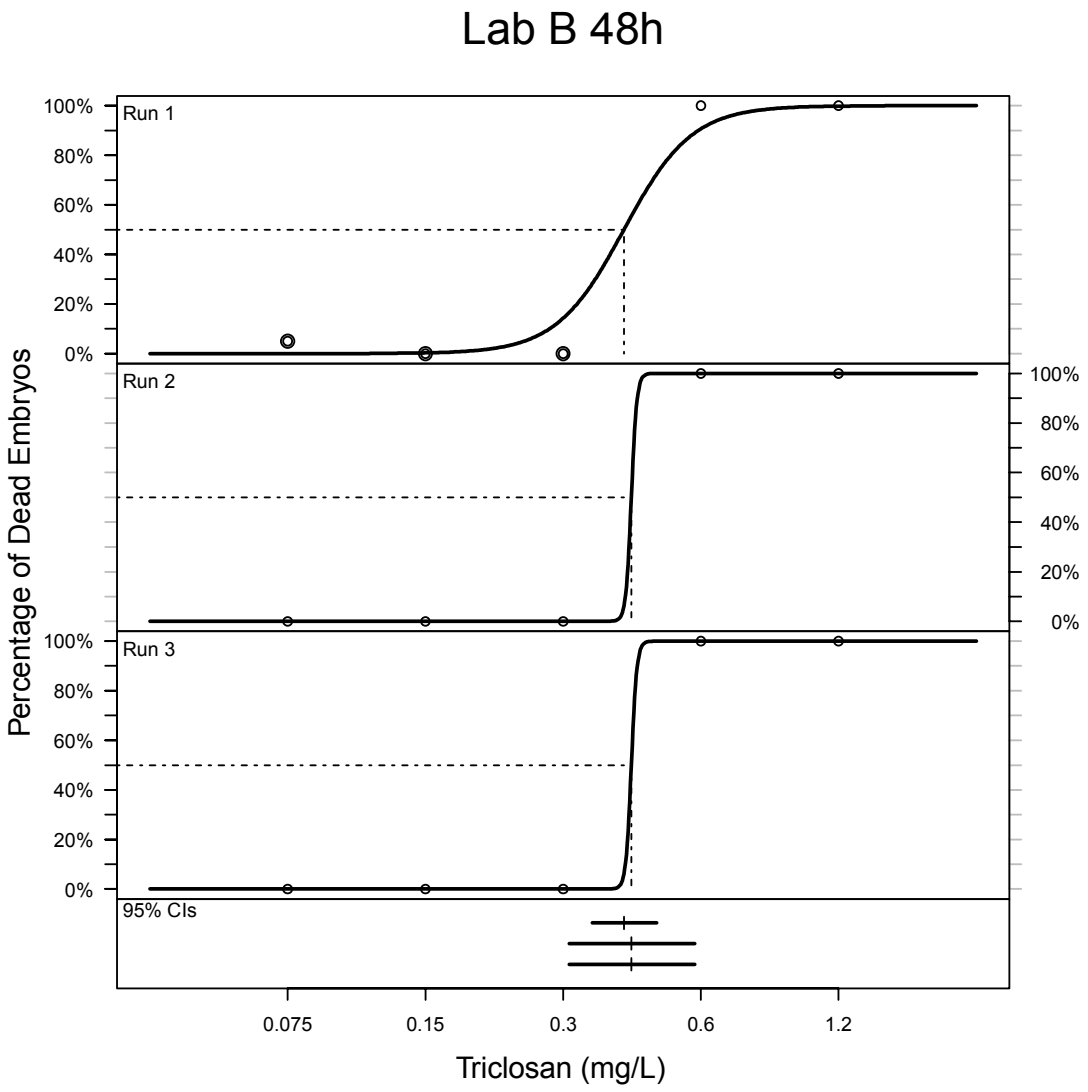
The in-practice application of this test method, like the one it aims to replace, is typically a single lab producing a single experimental result for a given chemical exposure. The experiment provides a single estimate of the LC50, and a confidence interval. For any of these methods, a single test result provides no estimate of the total variability in the estimate of the LC50 (e.g. experiment-to-experiment, lab-to-lab, and other factors such as water quality, health of embryos and so forth). Overall trends, however, are interpretable as seen in Figures 7 and 8. The chemicals included in Phase 1b span a wide range of toxicities, hydrophobicity, solubility and volatility and include "difficult substances" as defined in OECD Guidance Document 23[7]. The large degree of overlap in confidence intervals for a given chemical, the span of low to high EC50s, and reasonable Coefficients of Variation based on a small number of laboratories suggests the method is robust.

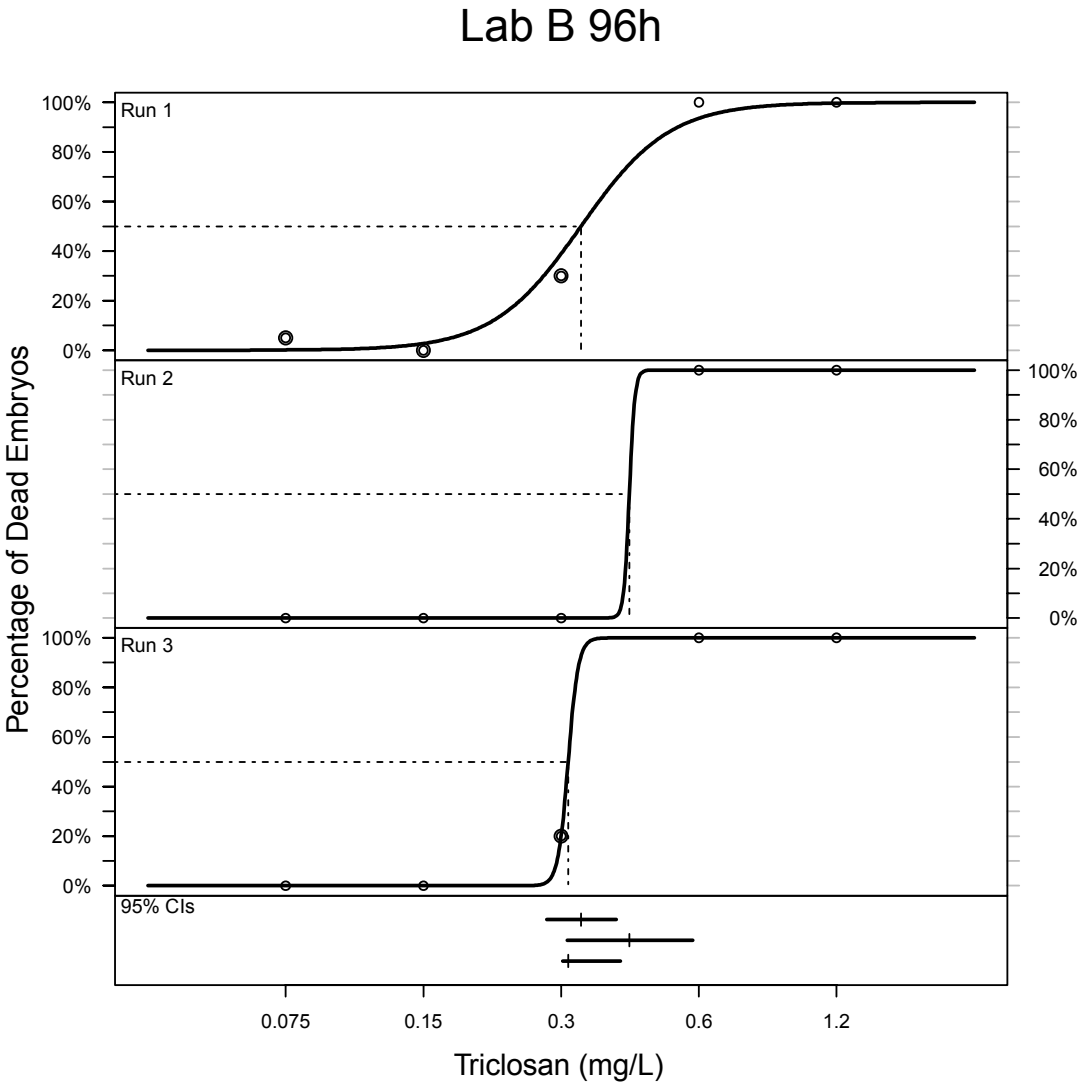
[7] OECD (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Series on Testing and Assessment, Number 23, Paris, France. 53p.

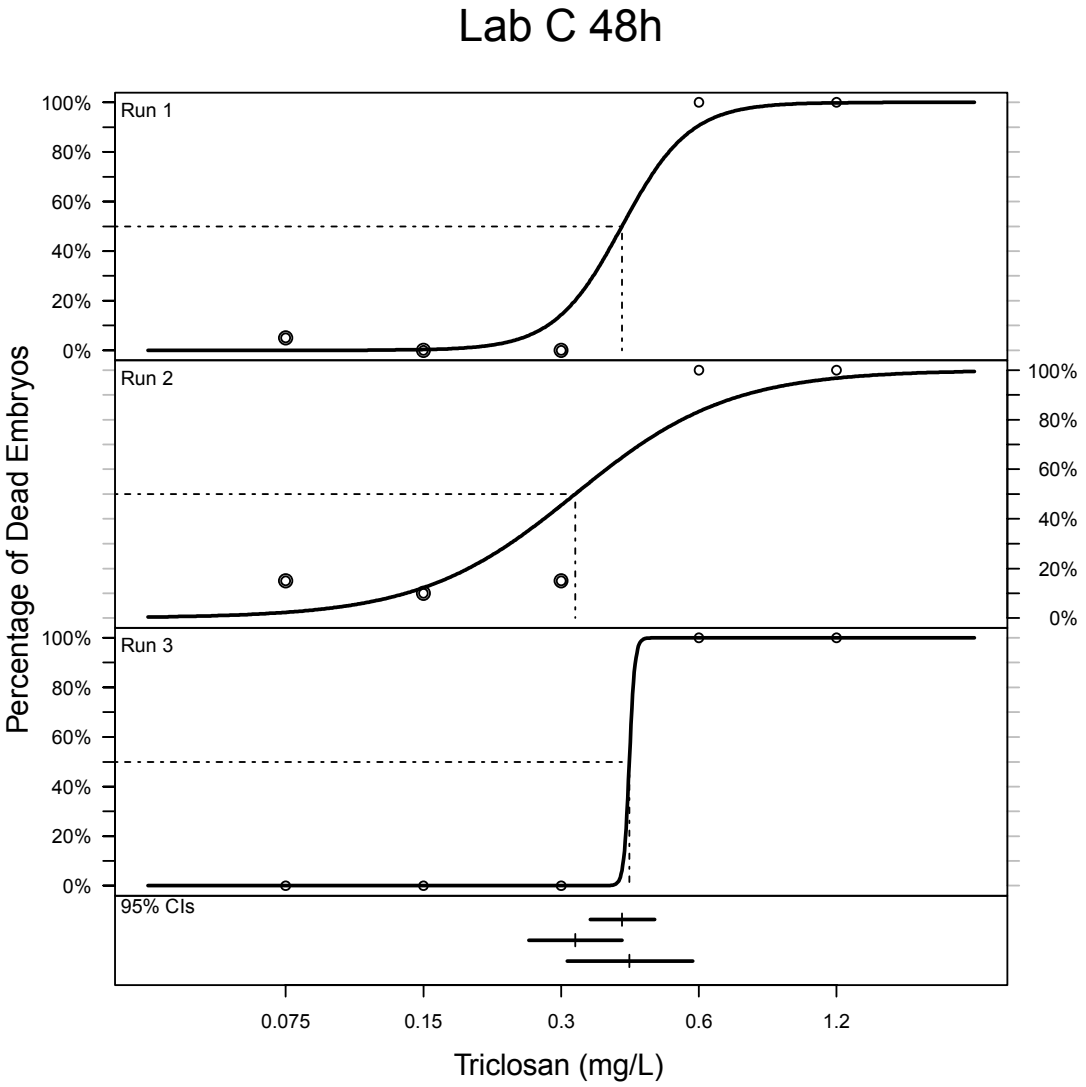
Appendix A: Phase 1b – Three Runs with six Chemicals

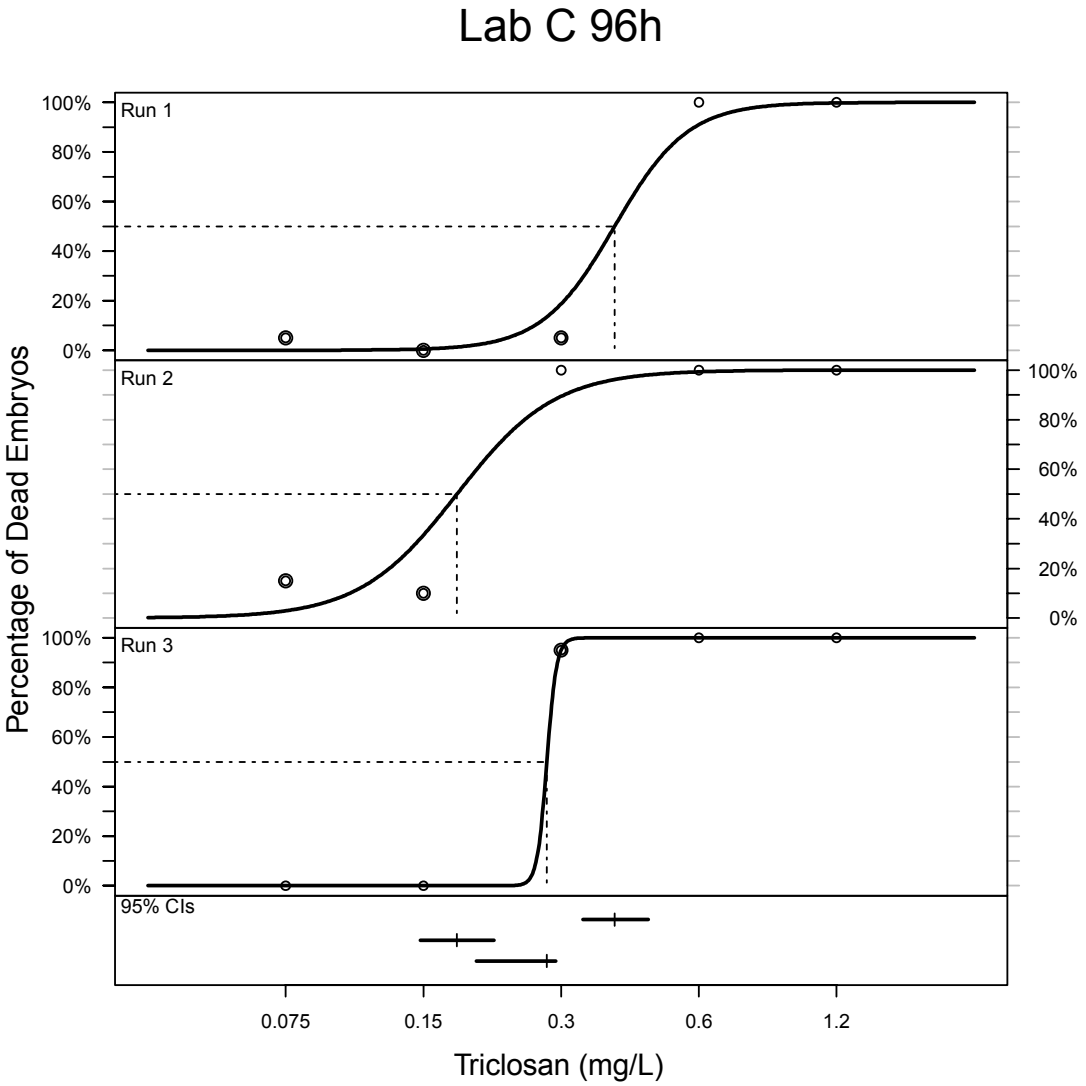
In each figure, the observed percentages of dead are the points and the prediction model is the solid curve. Dashed lines represent the estimated LC50, and associated confidence intervals are given in the lowest panel of each display. The larger points are the informative concentrations for fitting the curve. They are particularly important for estimating the slope of the prediction model. Ideally there will be two or more of these informative concentrations. When only one occurs, the slope will be arbitrarily steep very close to that concentration (e.g, run 1 immediately below), and when none occur (e.g, Triclosan Lab B 48h Run 2), the slope will be arbitrarily steep midway between the two concentrations that separate all 0% responses from all 100% responses.

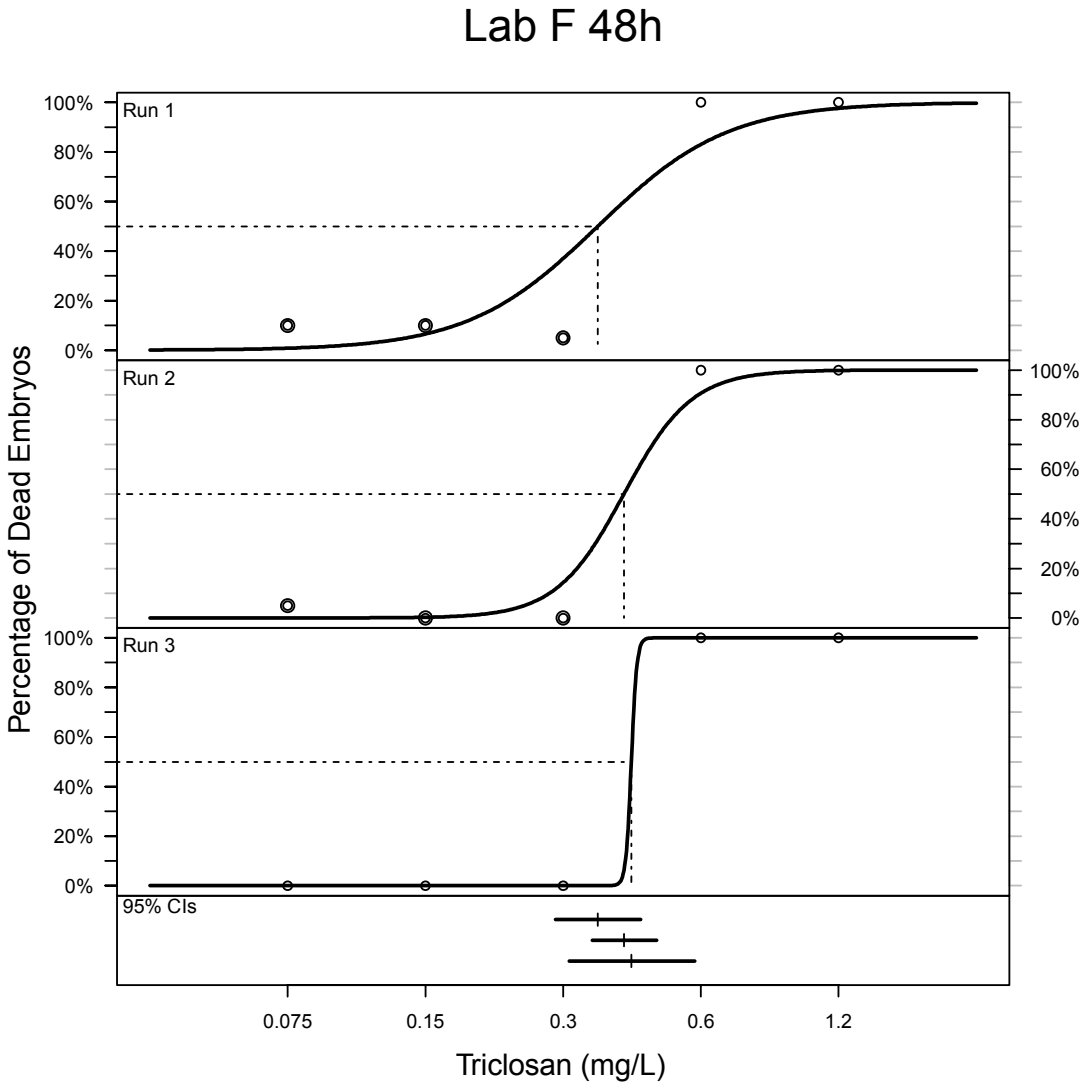
Triclosan

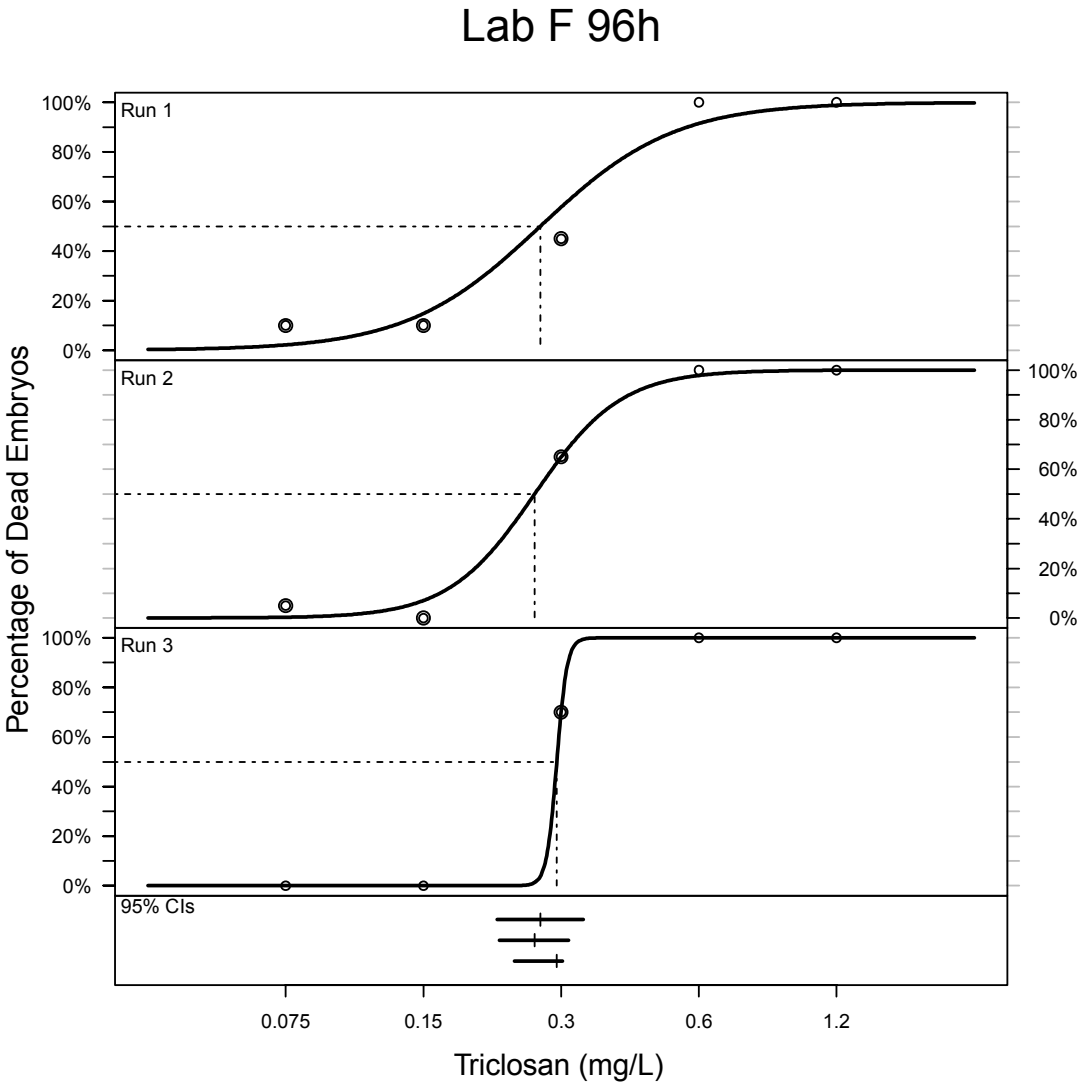


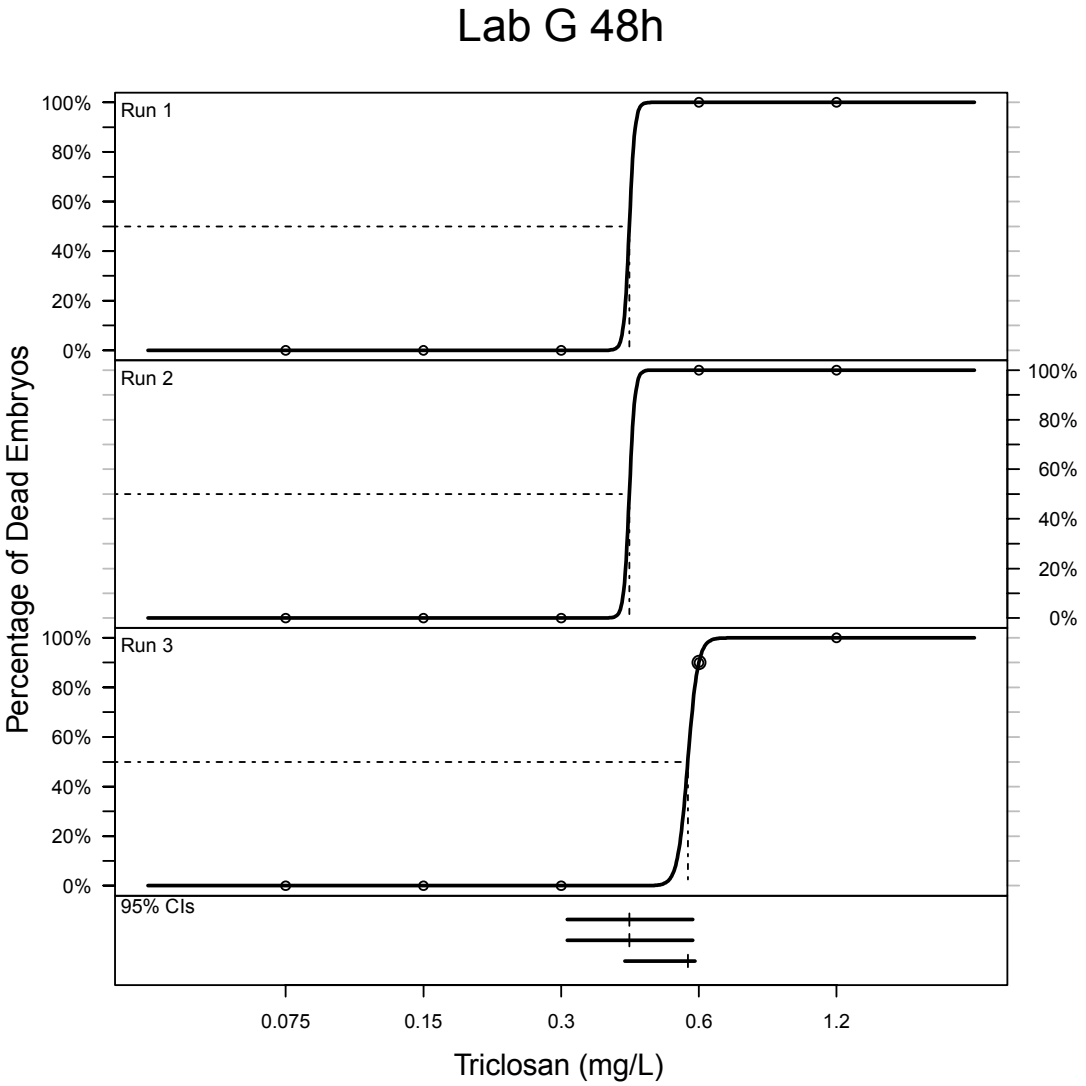

Lab B 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.075
0.15
0.3
0.6
1.2
Triclosan (mg/L)

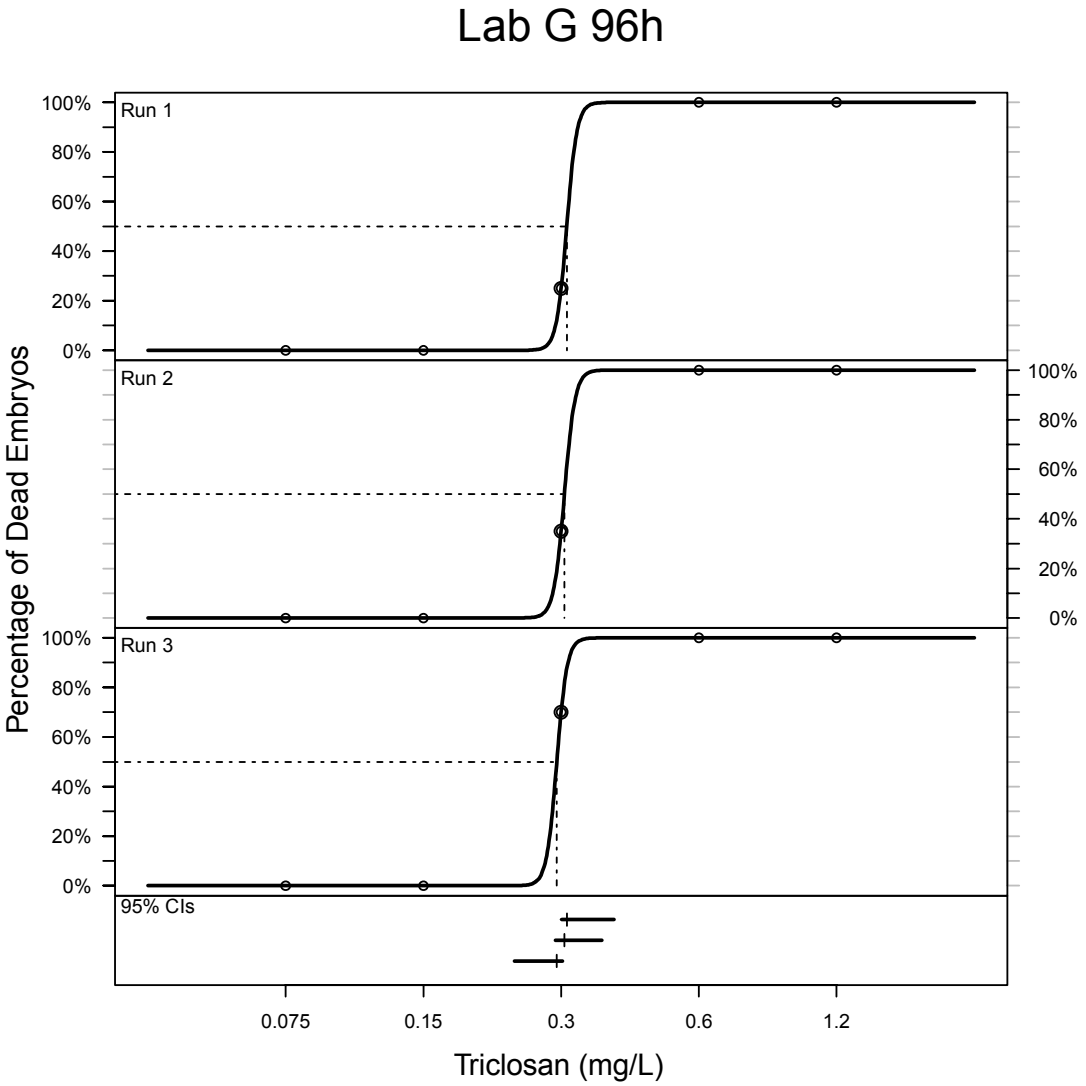

Lab C 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.075
0.15
0.3
0.6
1.2
Triclosan (mg/L)


Lab C 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.075
0.15
0.3
0.6
1.2
Triclosan (mg/L)

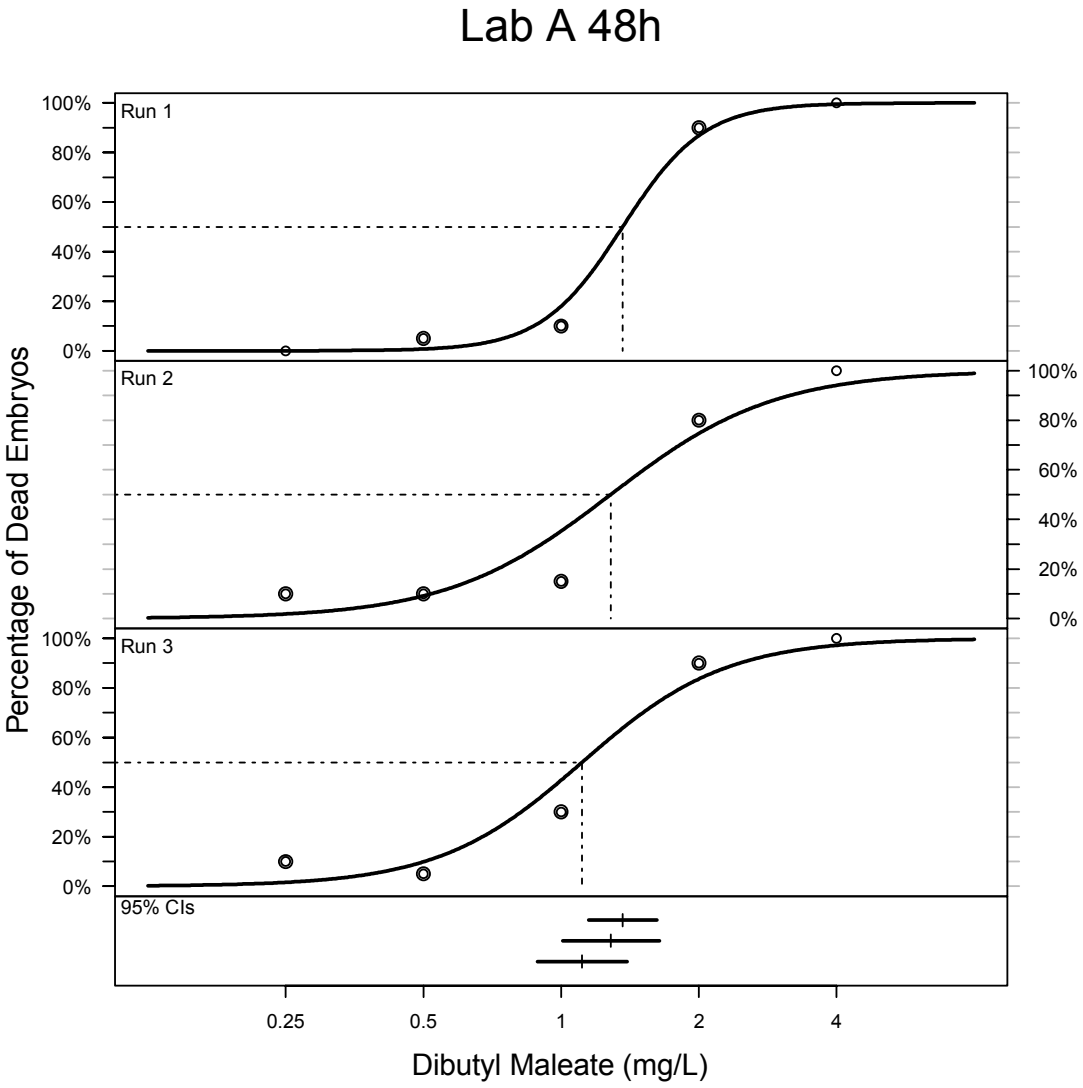

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.075
0.15
0.3
0.6
1.2
Triclosan (mg/L)

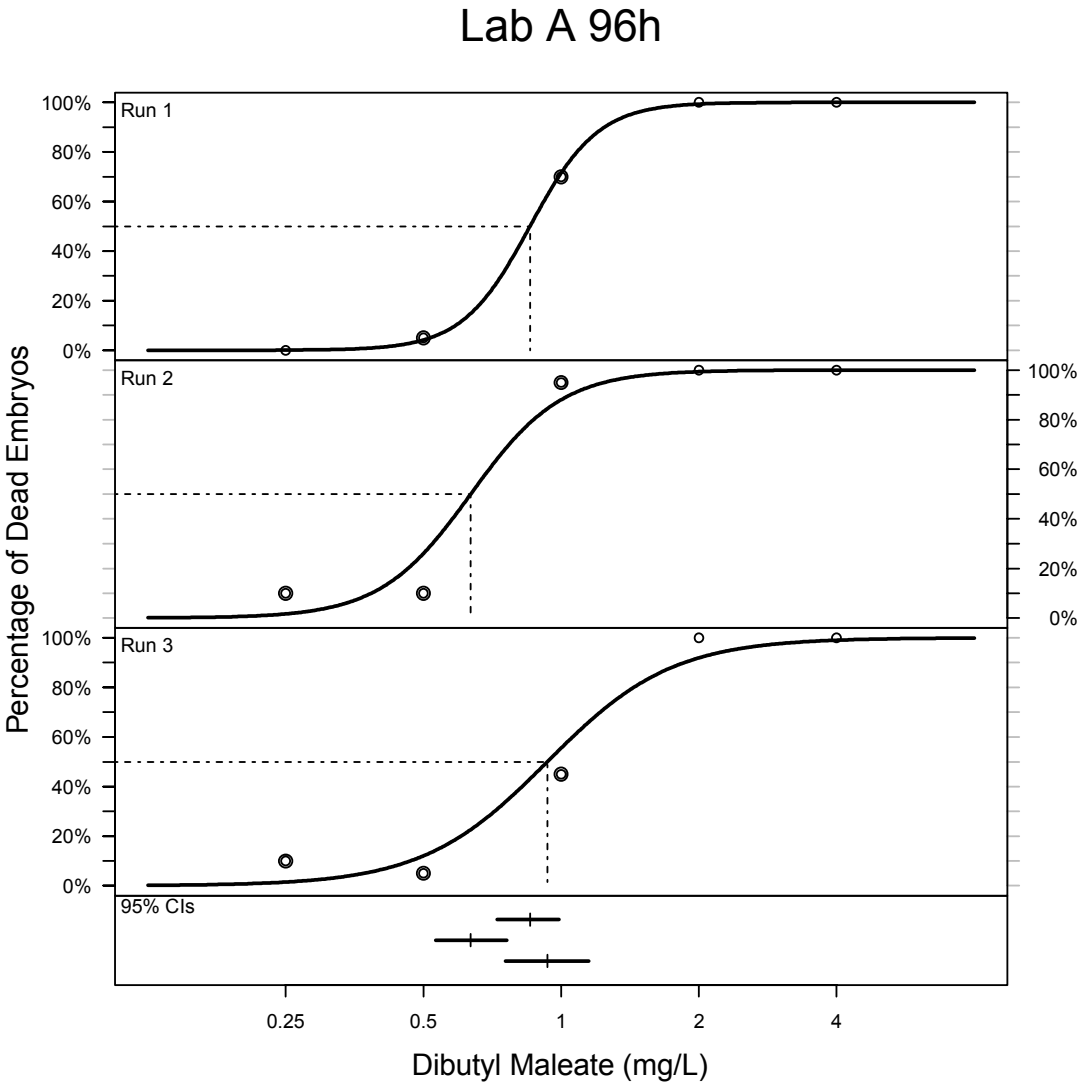

Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.075
0.15
0.3
0.6
1.2
Triclosan (mg/L)

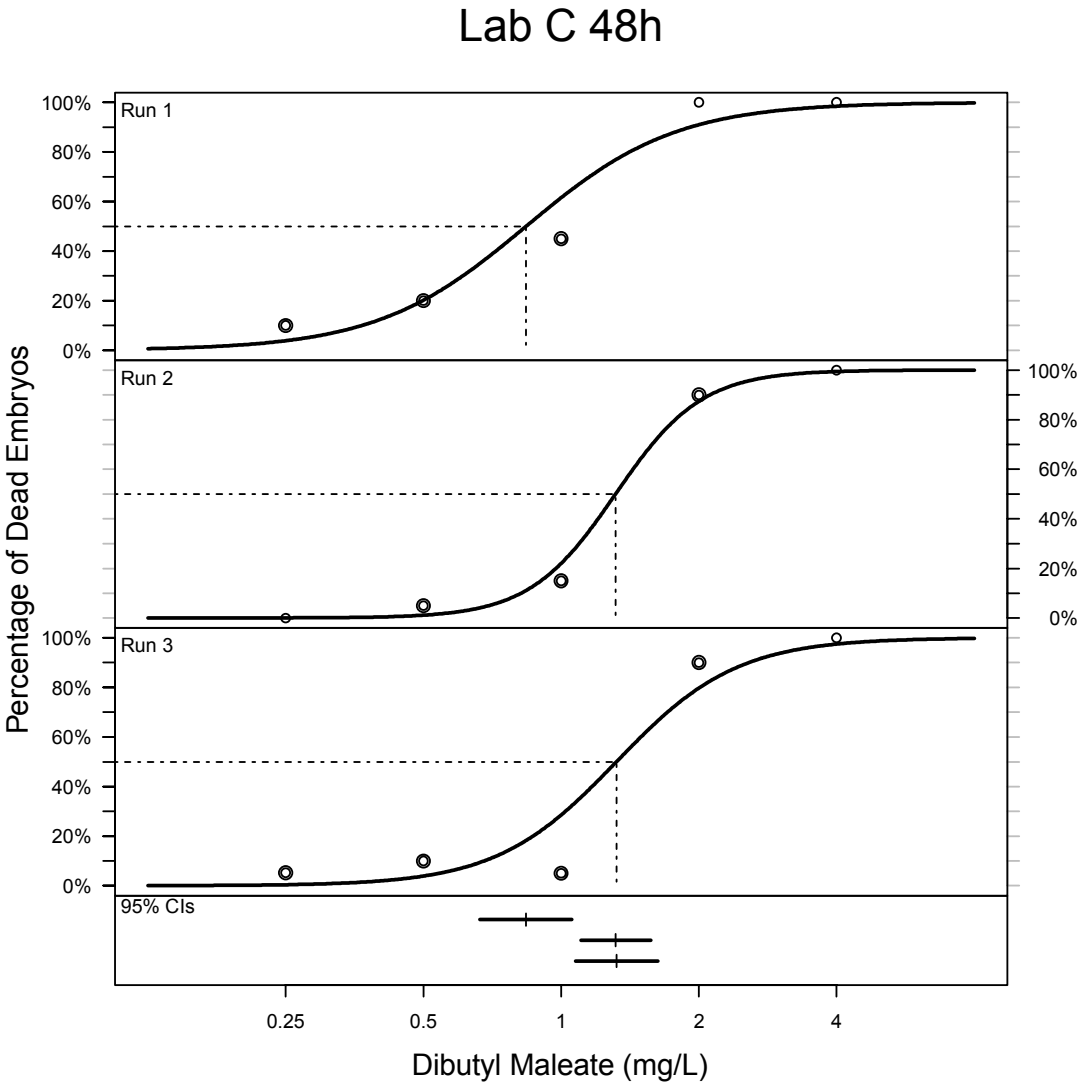

Lab G 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.075
0.15
0.3
0.6
1.2
Triclosan (mg/L)

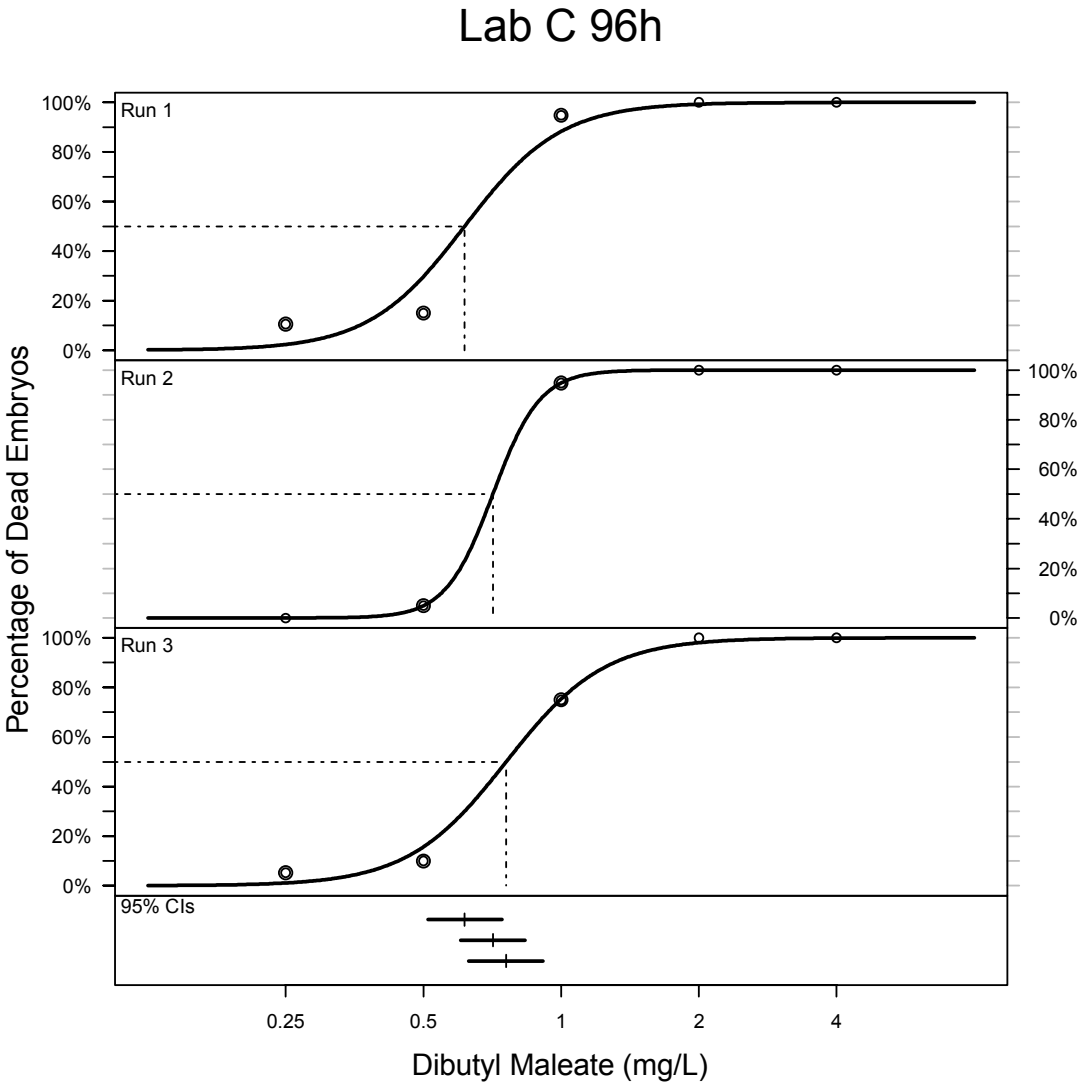

Lab G 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.075
0.15
0.3
0.6
1.2
Triclosan (mg/L)

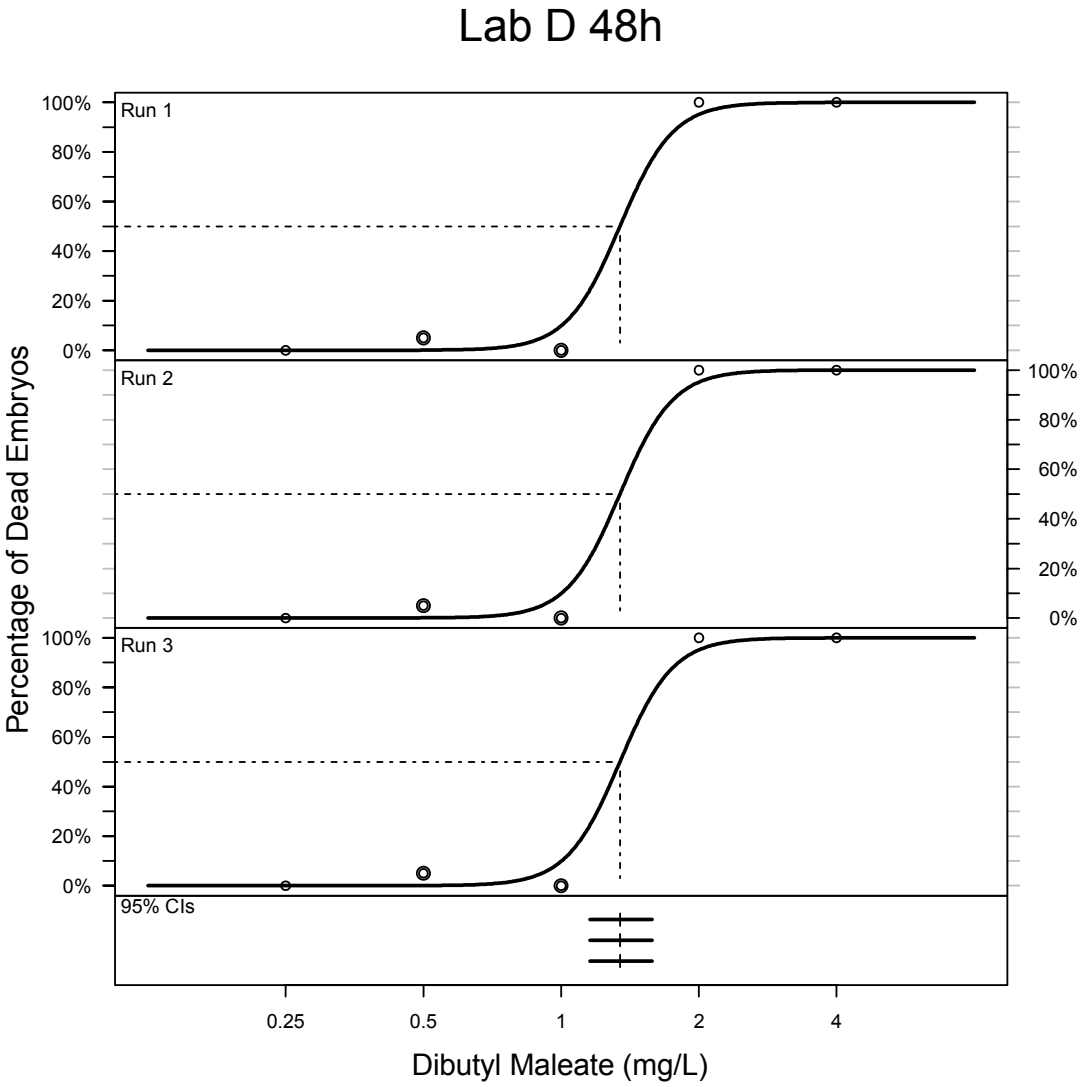
Dibutyl Maleate

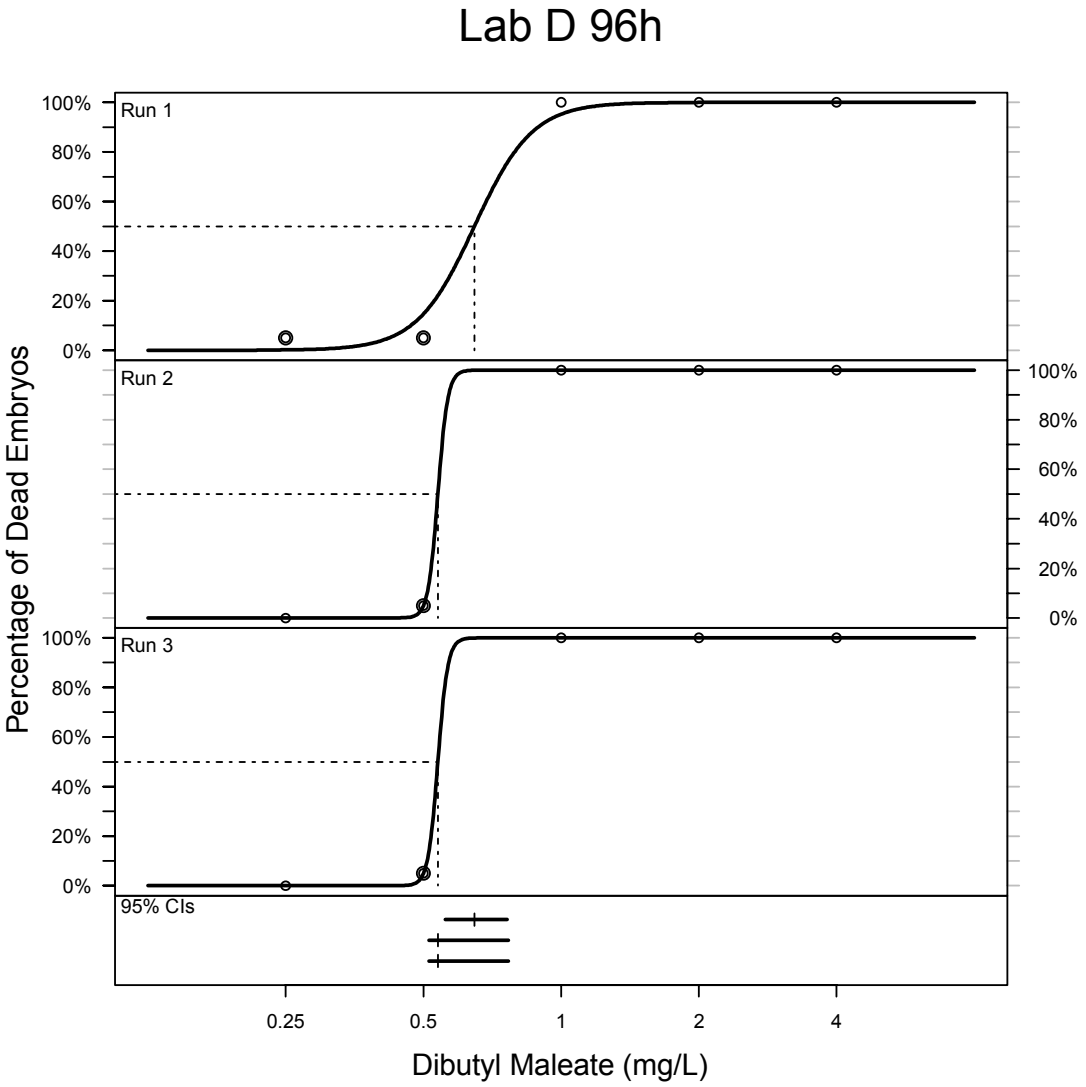


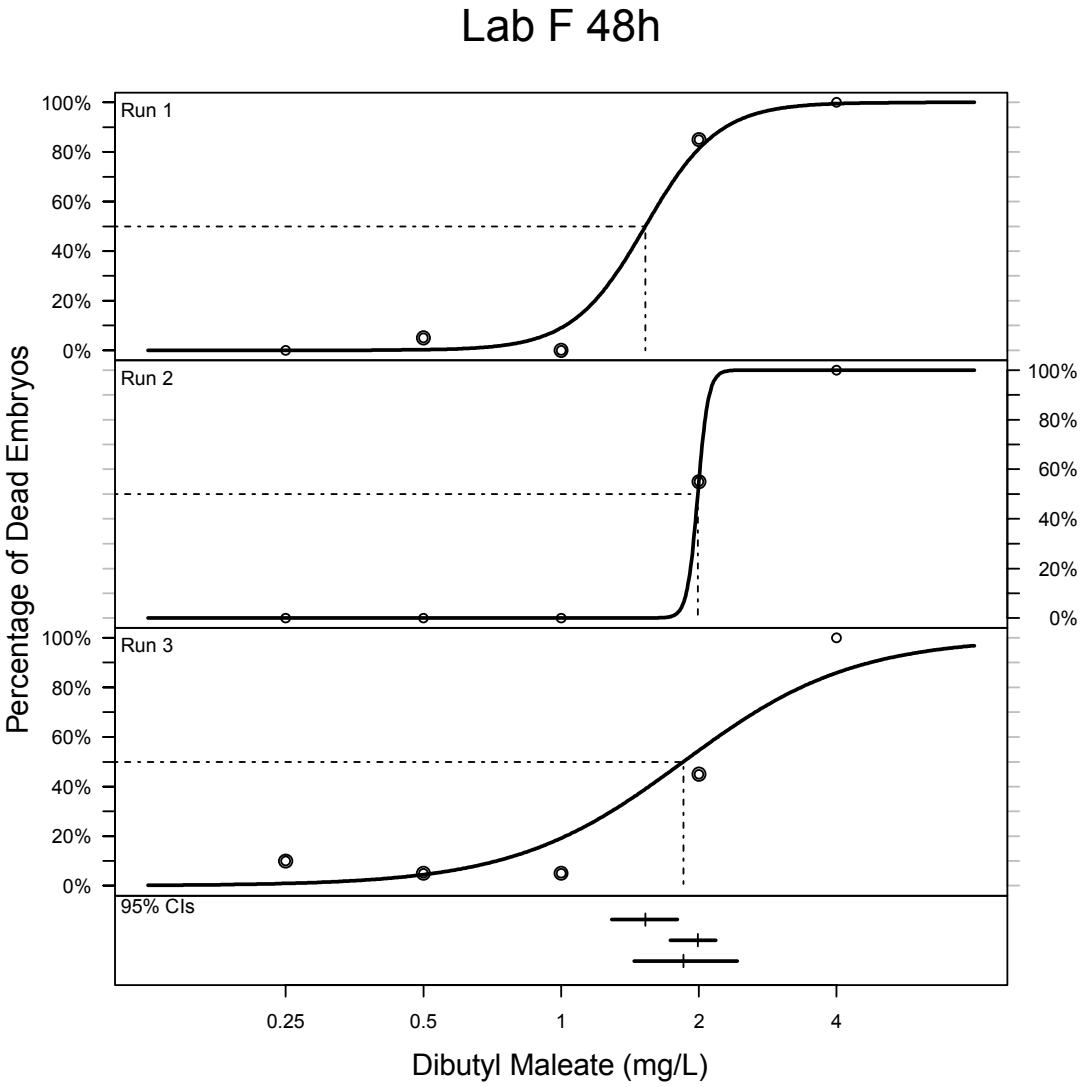

Lab A 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.25
0.5
1
2
4
Dibutyl Maleate (mg/L)

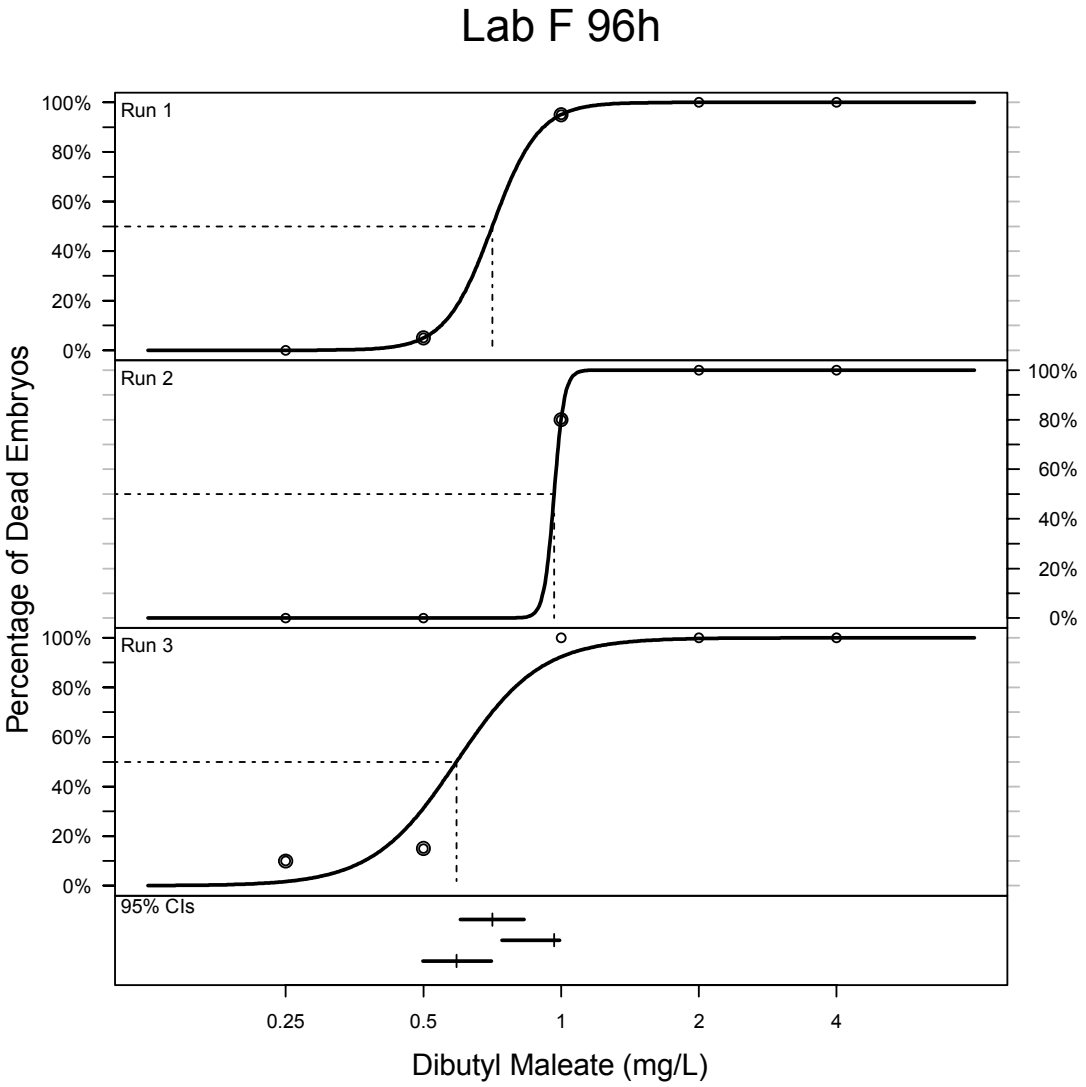

Lab C 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.25
0.5
1
2
4
Dibutyl Maleate (mg/L)

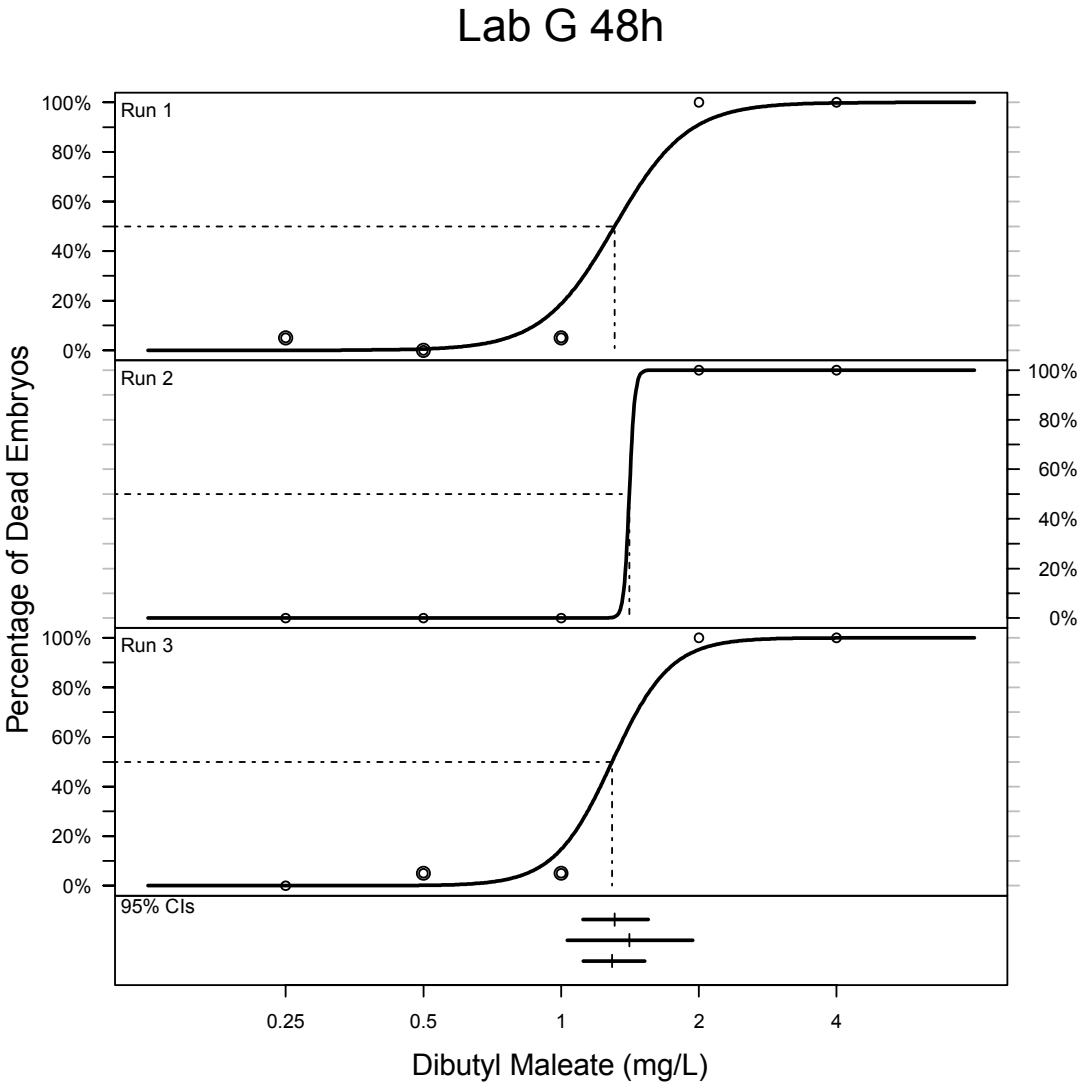

Lab C 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.25
0.5
1
2
4
Dibutyl Maleate (mg/L)

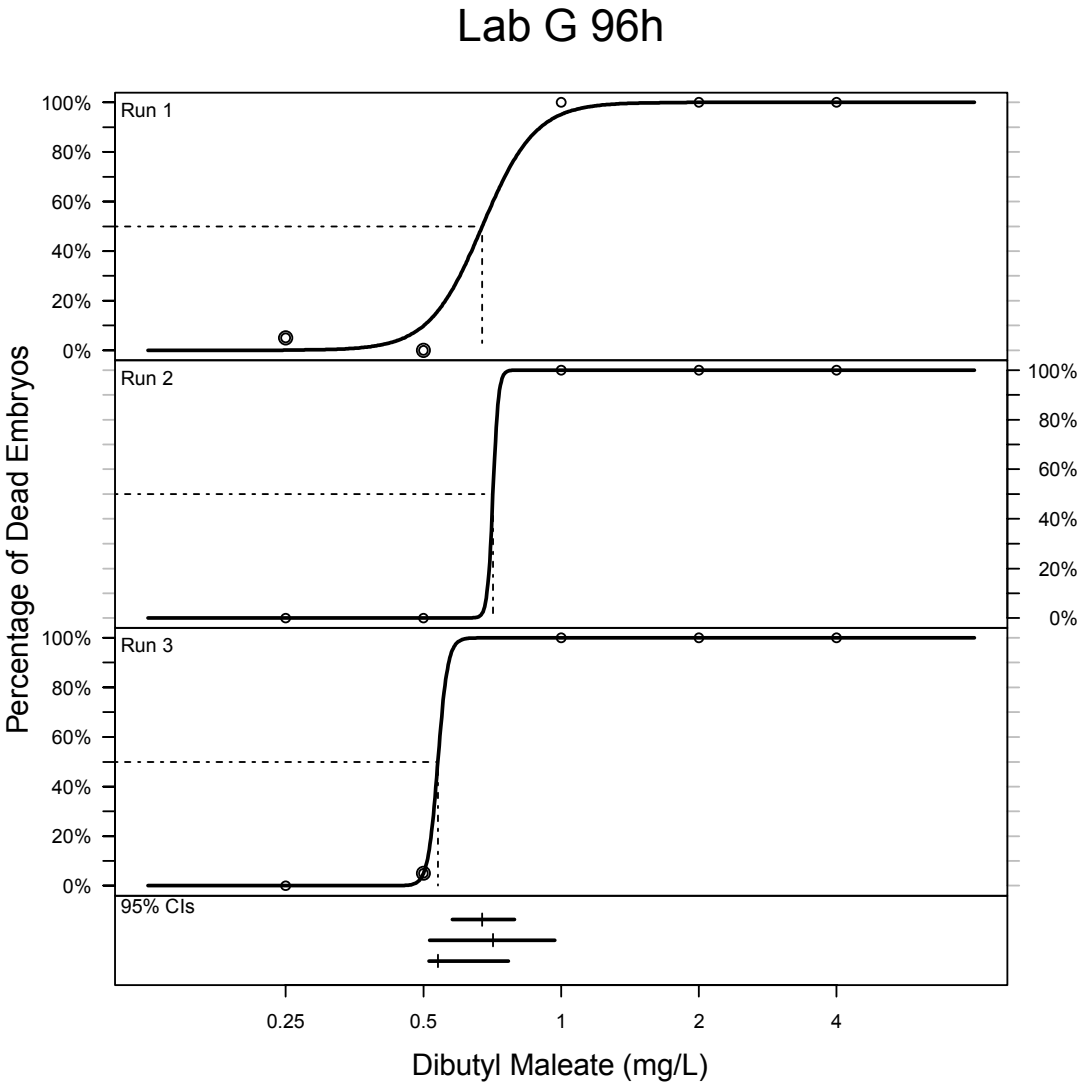

Lab D 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.25
0.5
1
2
4
Dibutyl Maleate (mg/L)


Lab D 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.25
0.5
1
2
4
Dibutyl Maleate (mg/L)

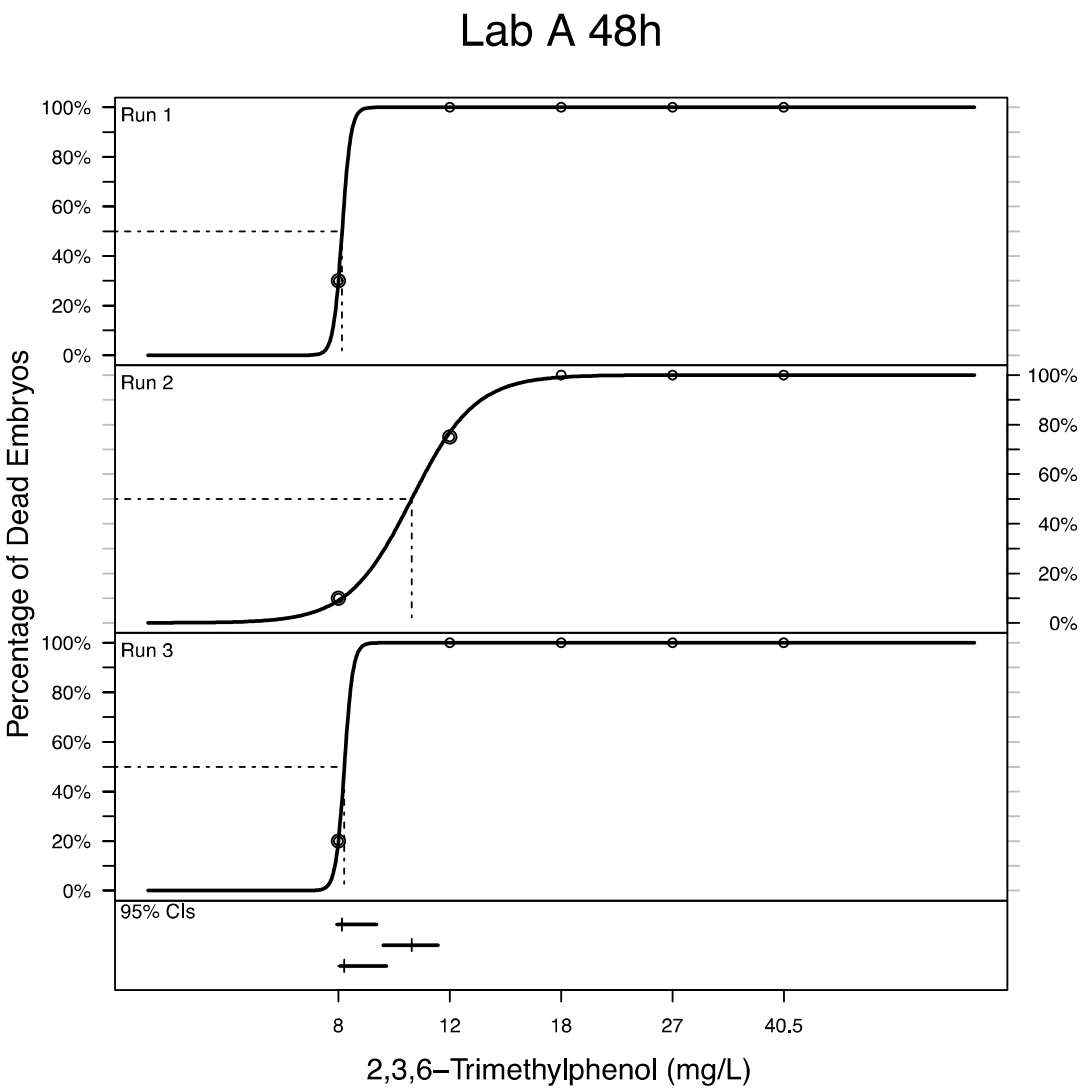

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.25
0.5
1
2
4
Dibutyl Maleate (mg/L)

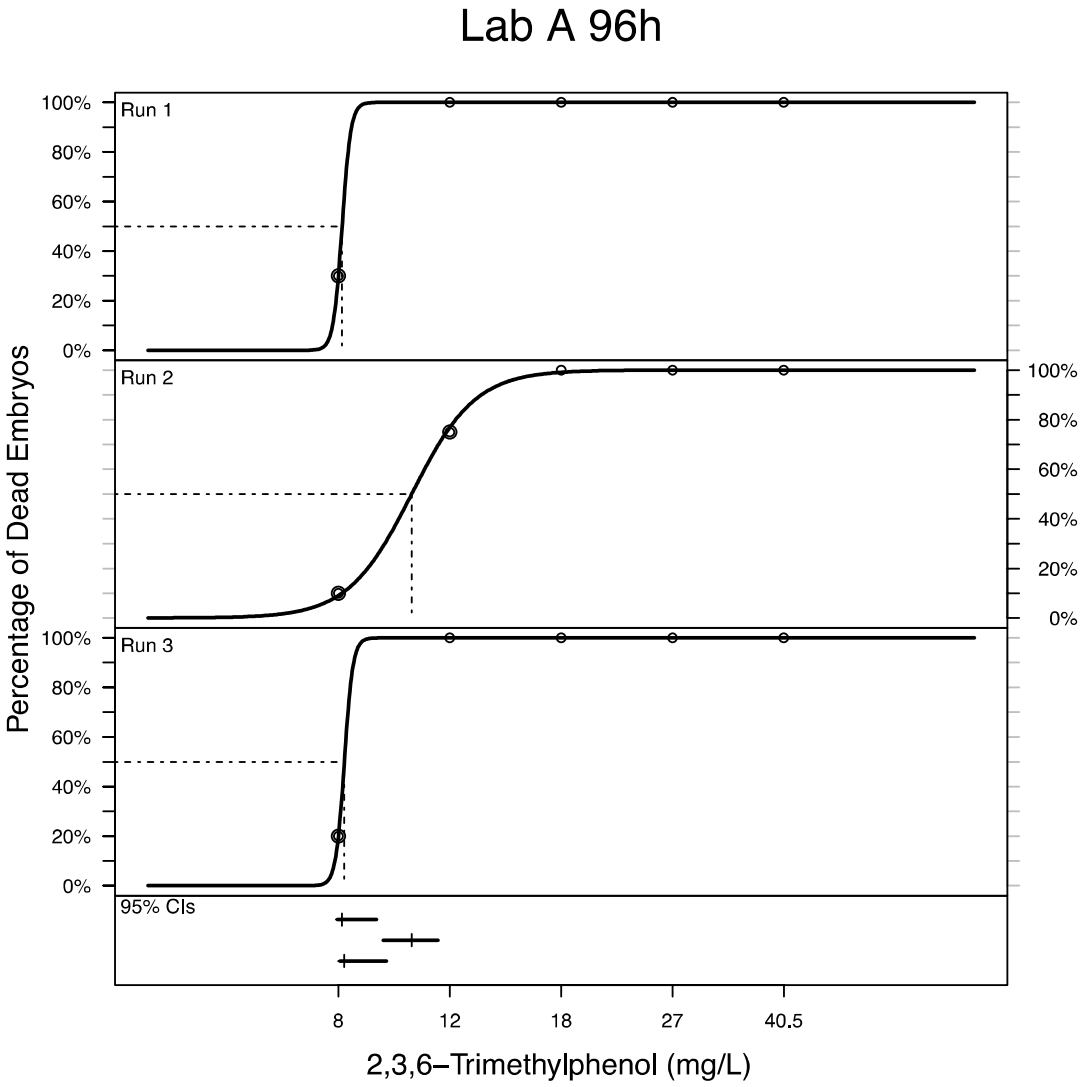

Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.25
0.5
1
2
4
Dibutyl Maleate (mg/L)

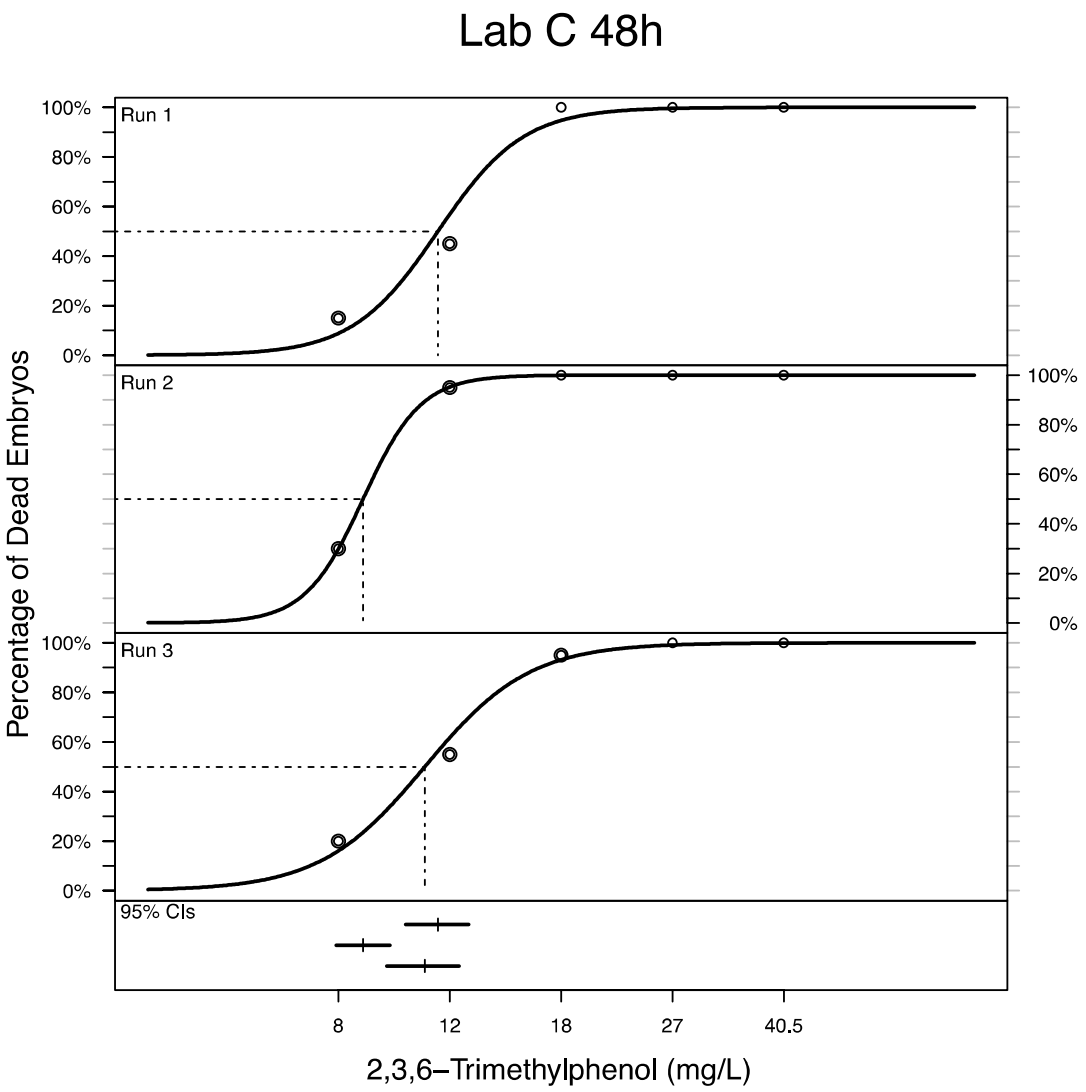

Lab G 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.25
0.5
1
2
4
Dibutyl Maleate (mg/L)

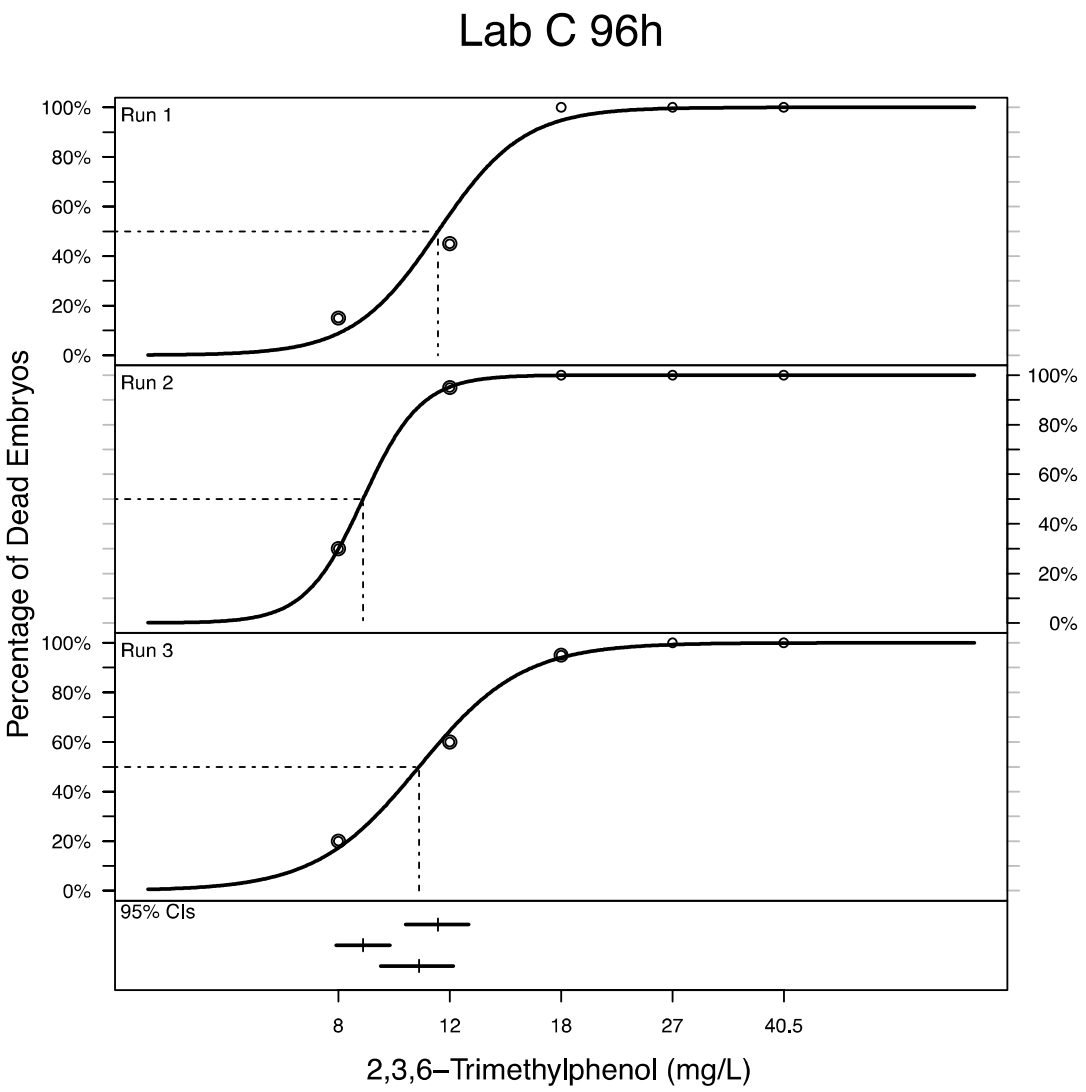

Lab G 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
0.25
0.5
1
2
4
Dibutyl Maleate (mg/L)

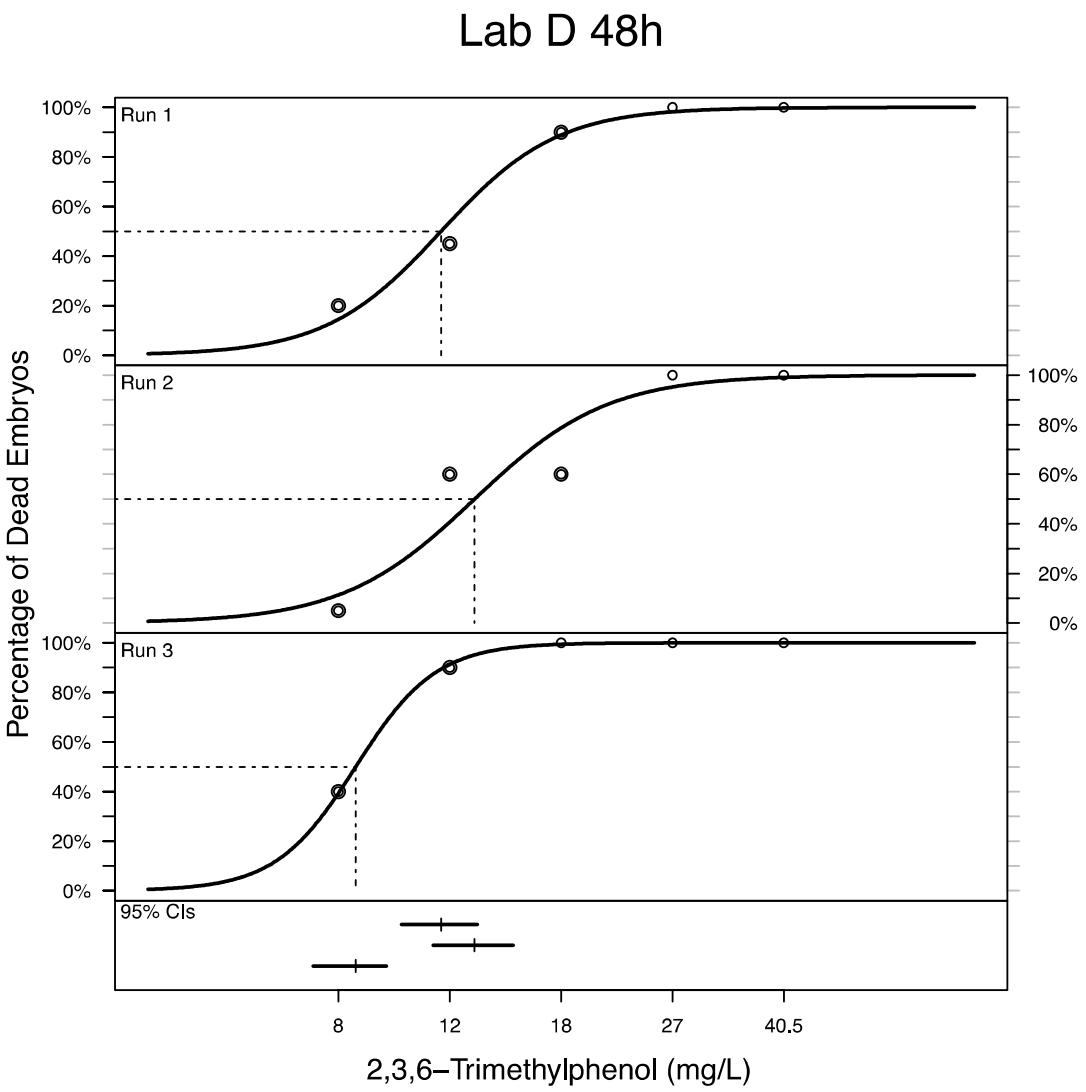
2,3,6-Trimethylphenol

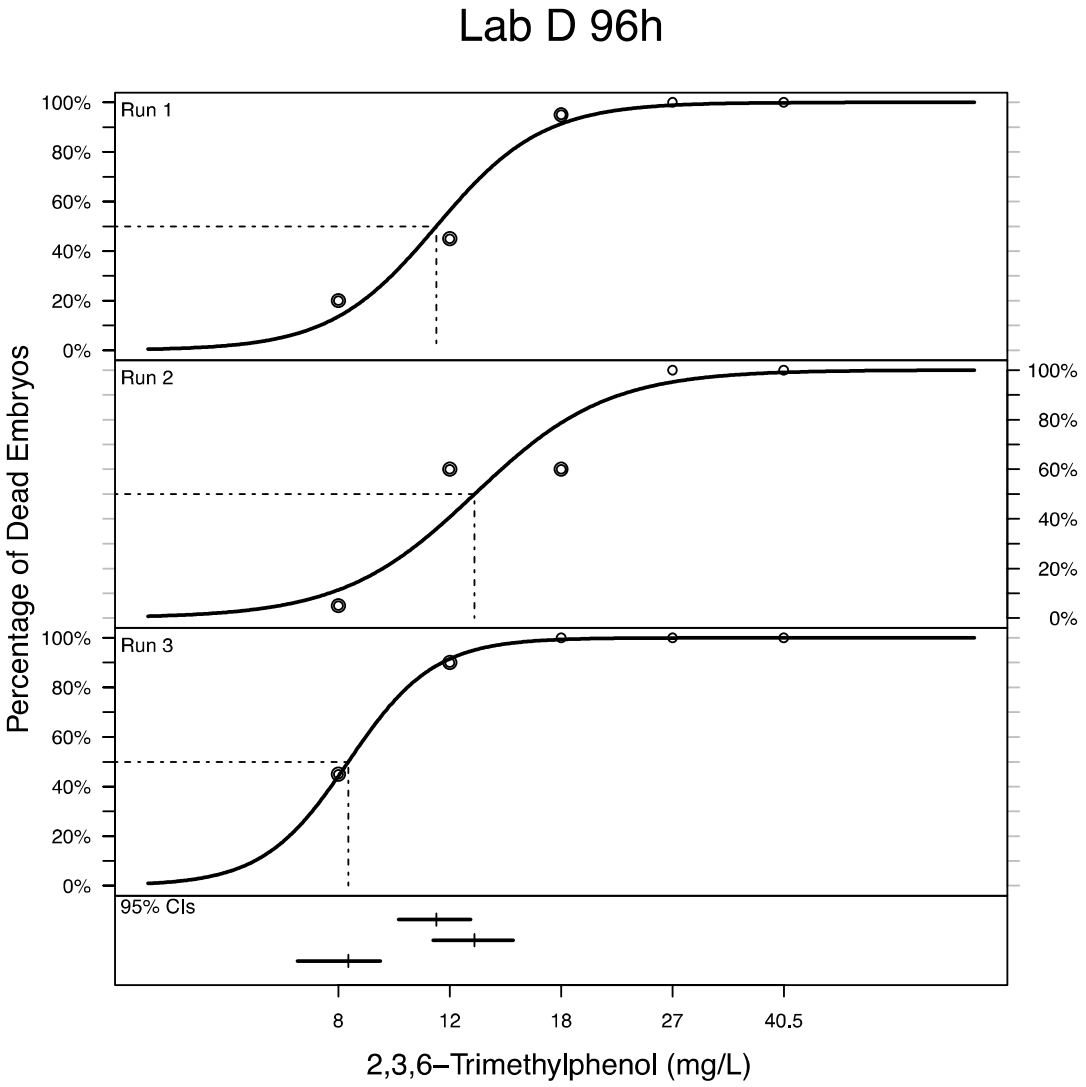


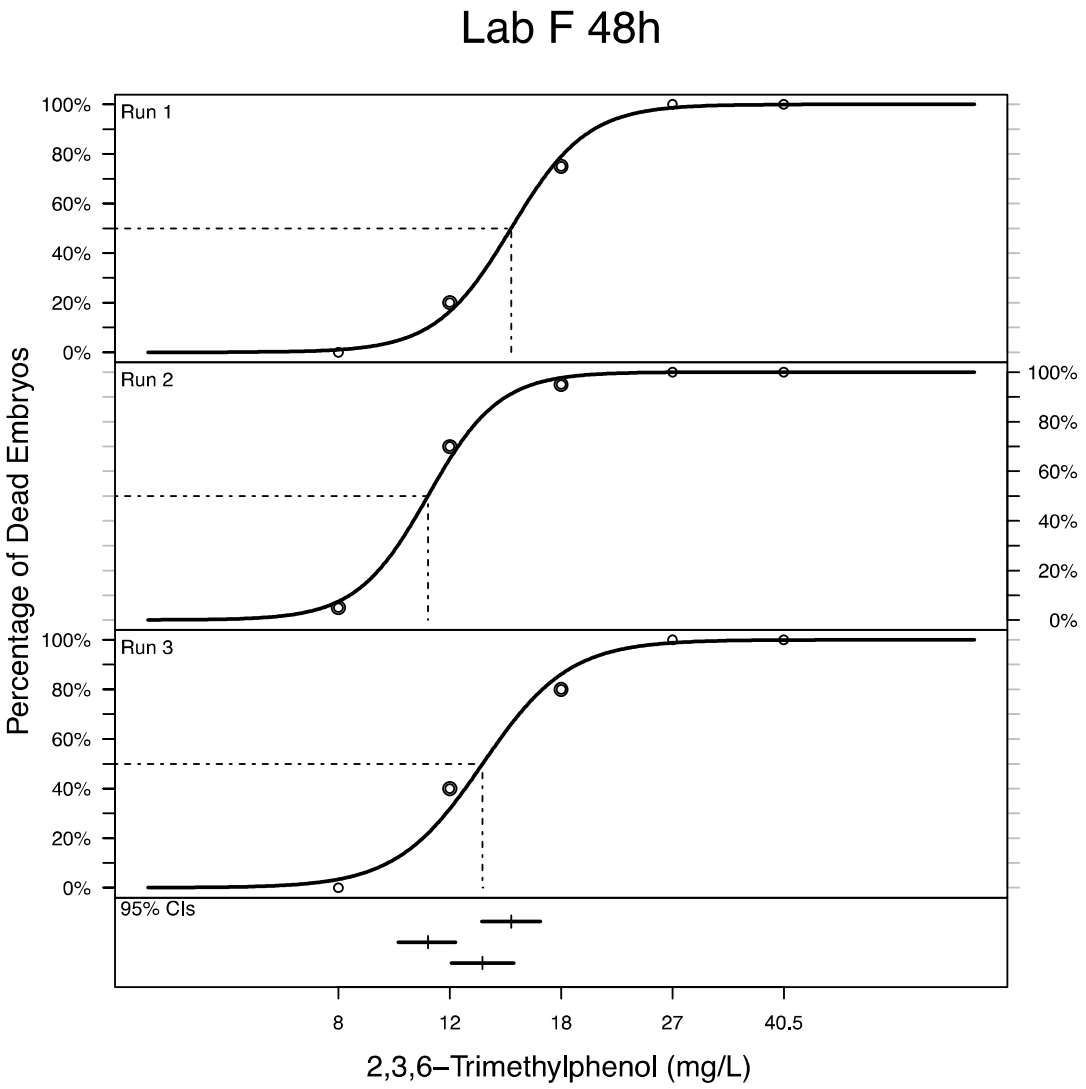

Lab A 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
8
12
18
27
40.5
2,3,6–Trimethylphenol (mg/L)

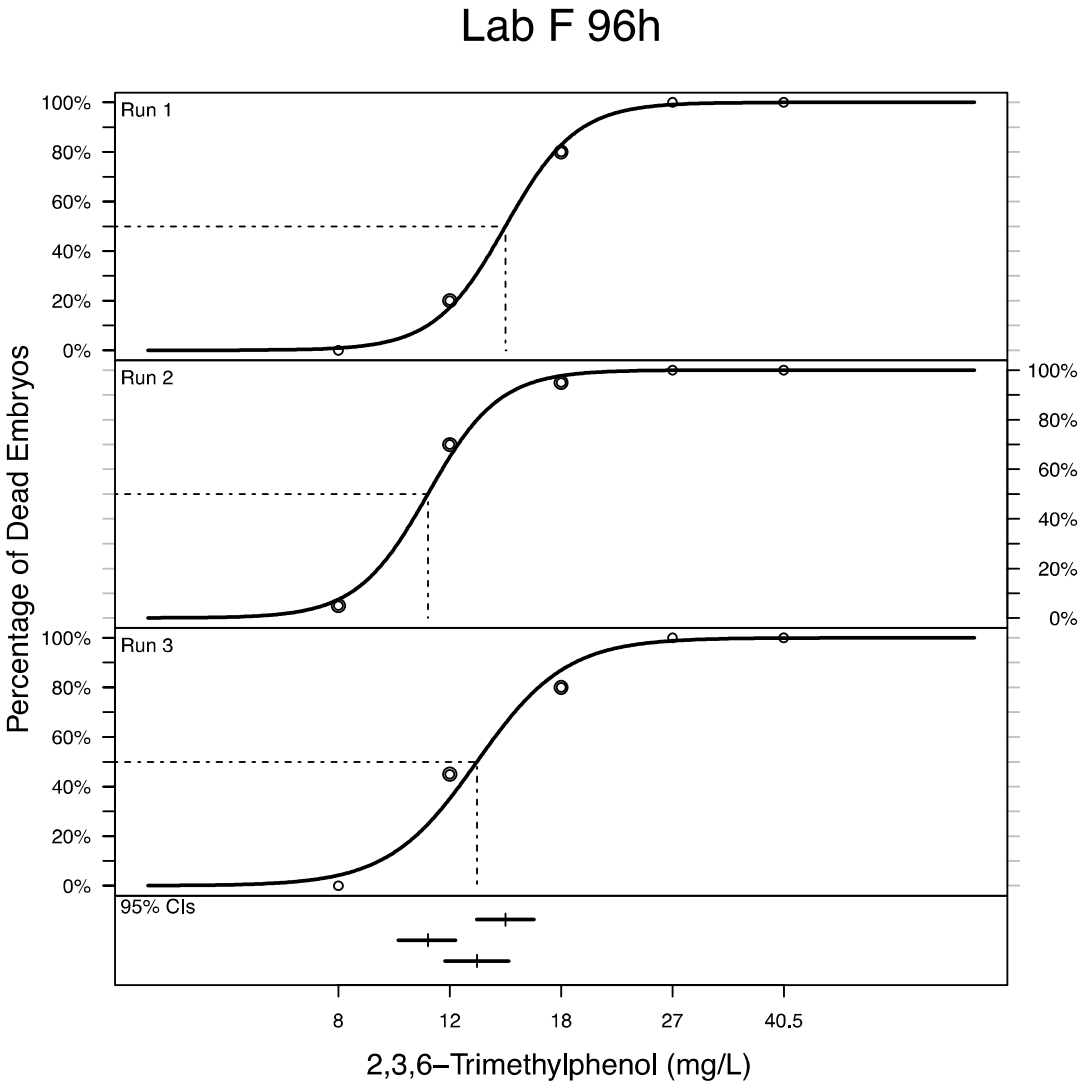

Lab C 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
8
12
18
27
40.5
2,3,6–Trimethylphenol (mg/L)

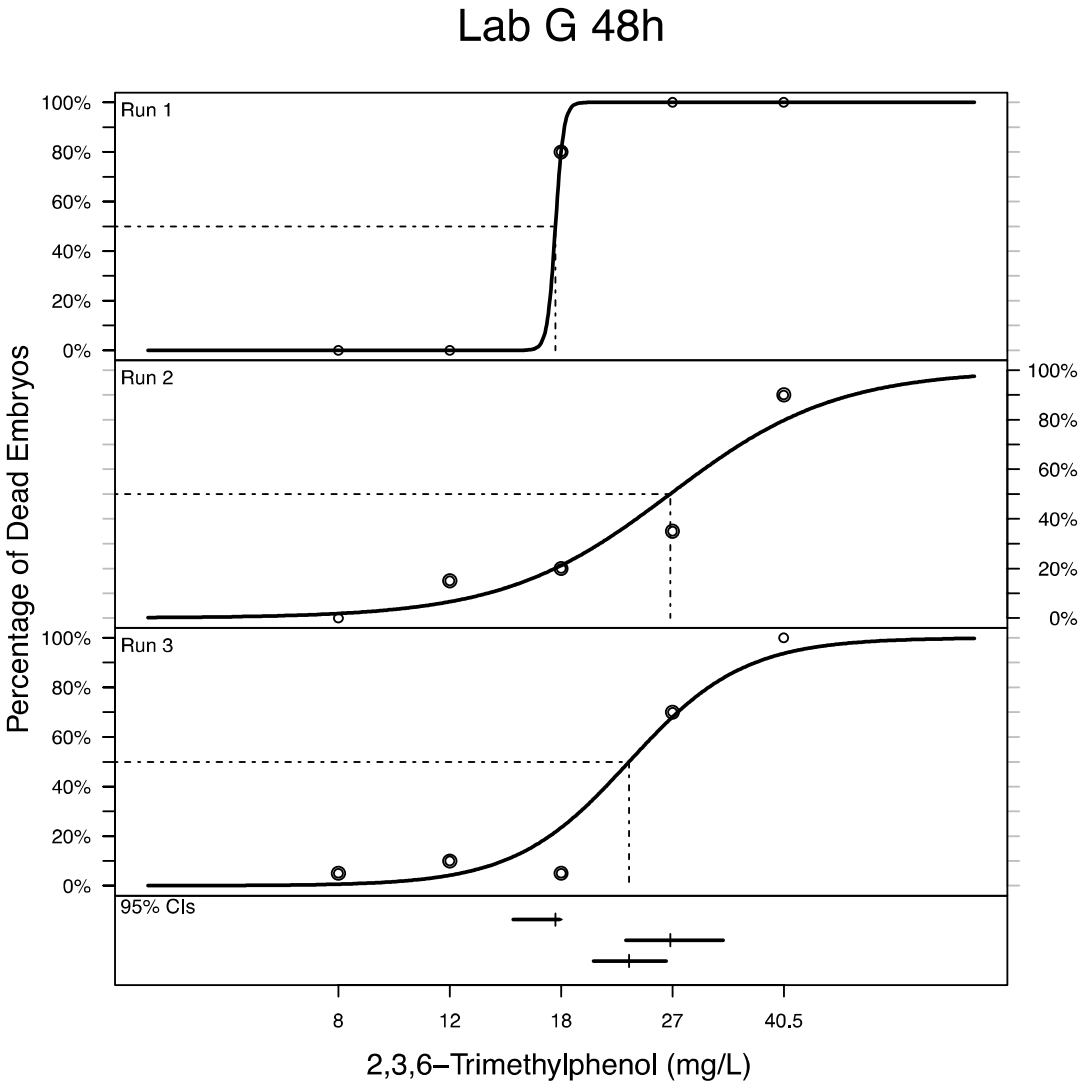

Lab C 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
8
12
18
27
40.5
2,3,6–Trimethylphenol (mg/L)

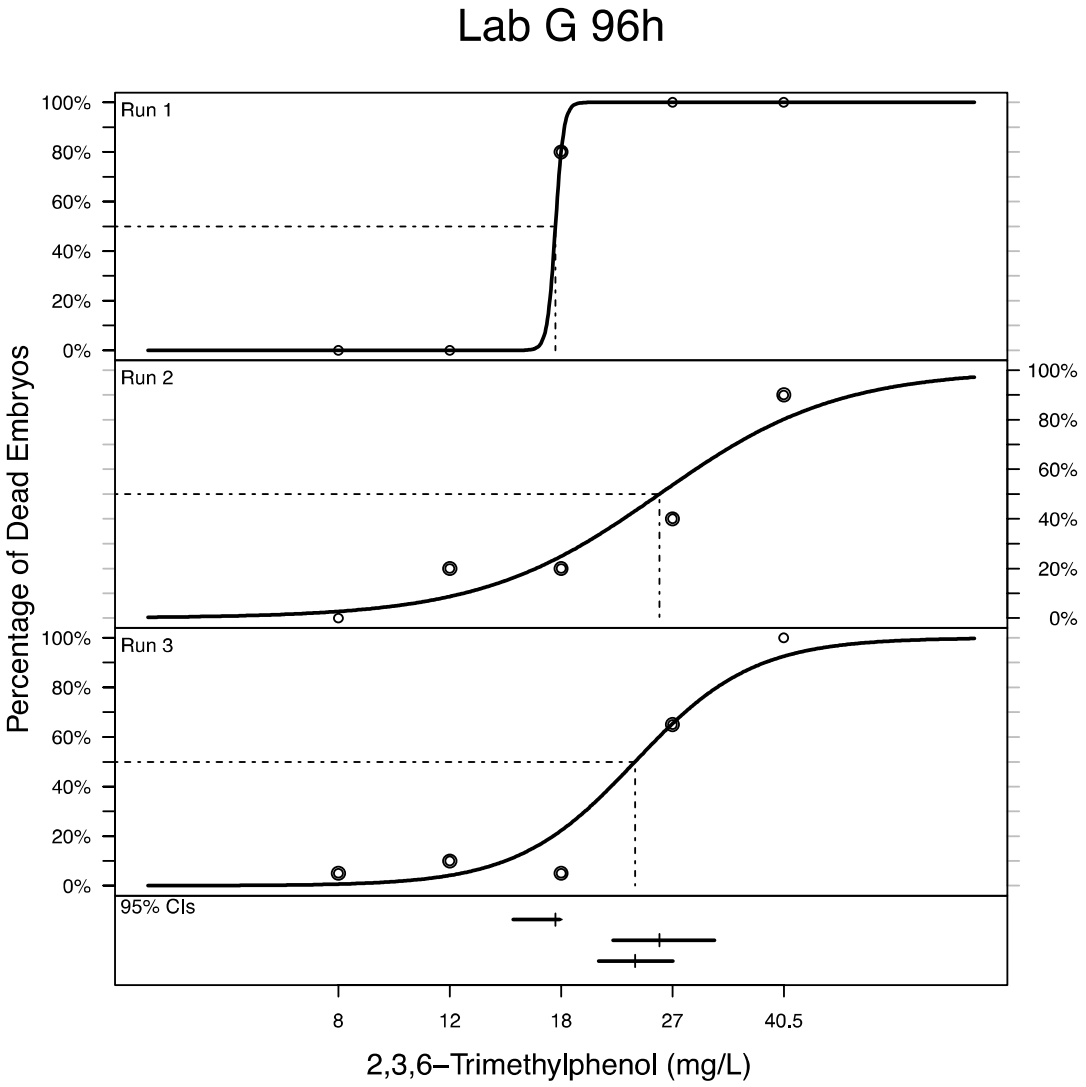

Lab D 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
8
12
18
27
40.5
2,3,6–Trimethylphenol (mg/L)


Lab D 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
8
12
18
27
40.5
2,3,6–Trimethylphenol (mg/L)

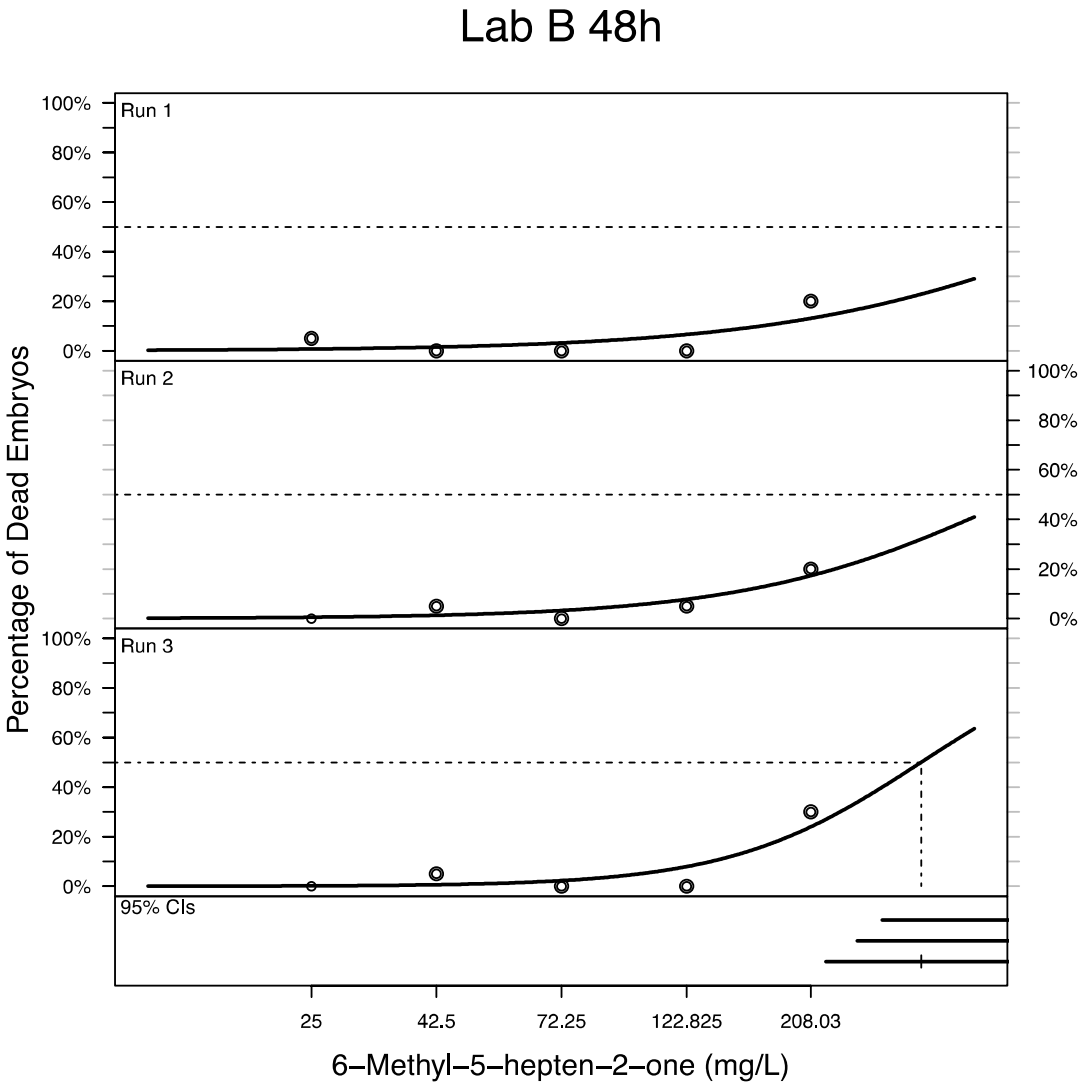

Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
8
12
18
27
40.5
2,3,6–Trimethylphenol (mg/L)

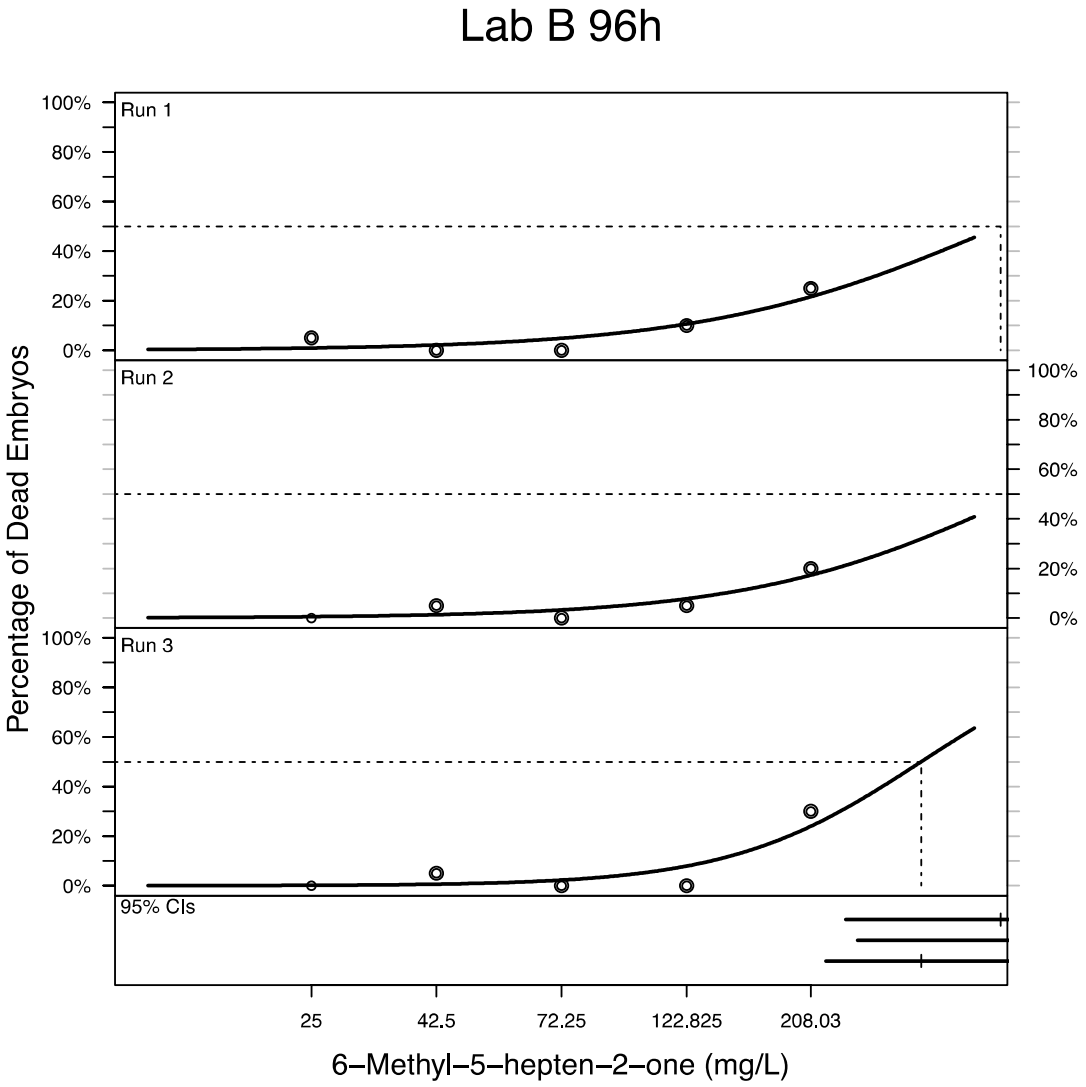

Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
8
12
18
27
40.5
2,3,6–Trimethylphenol (mg/L)

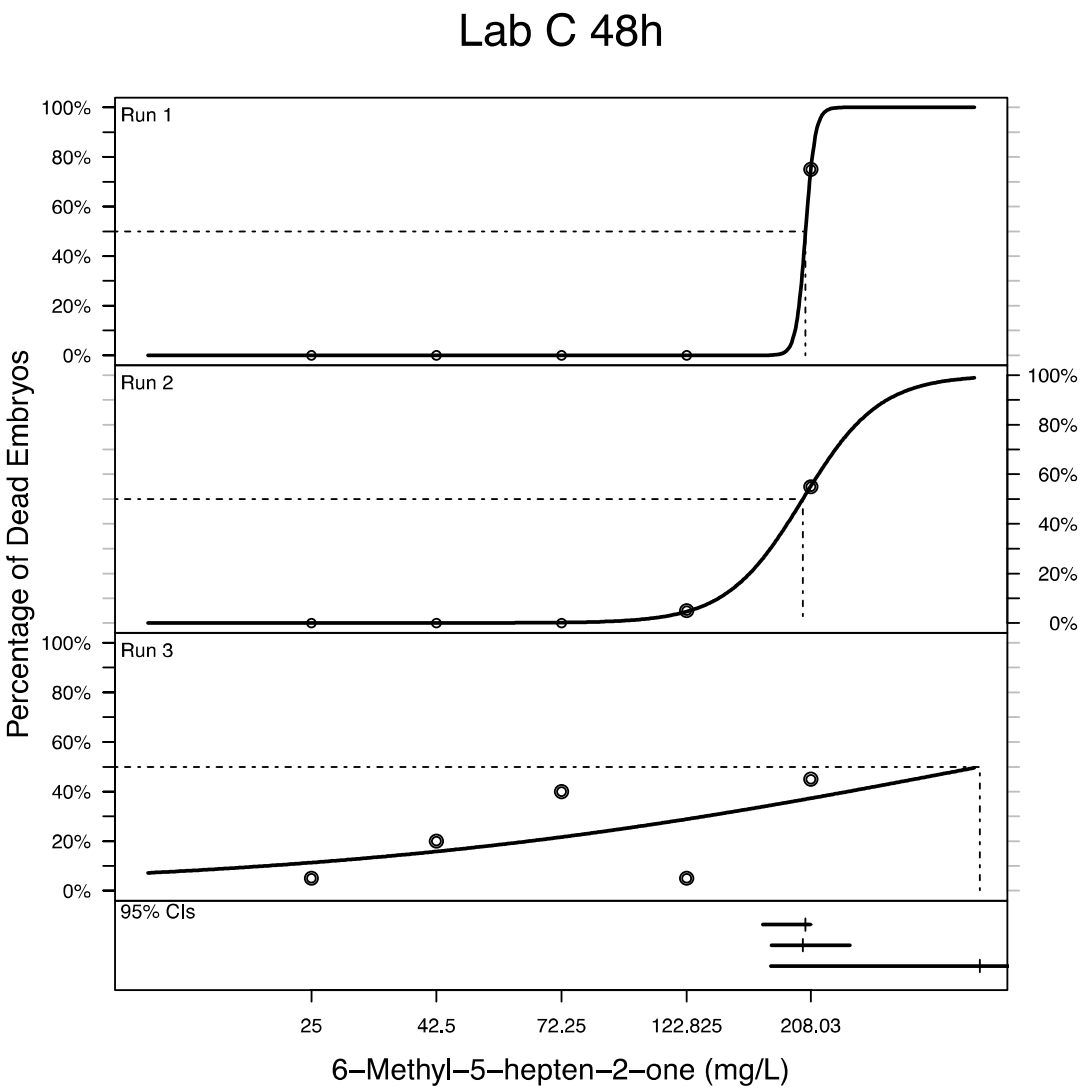

Lab G 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
8
12
18
27
40.5
2,3,6–Trimethylphenol (mg/L)

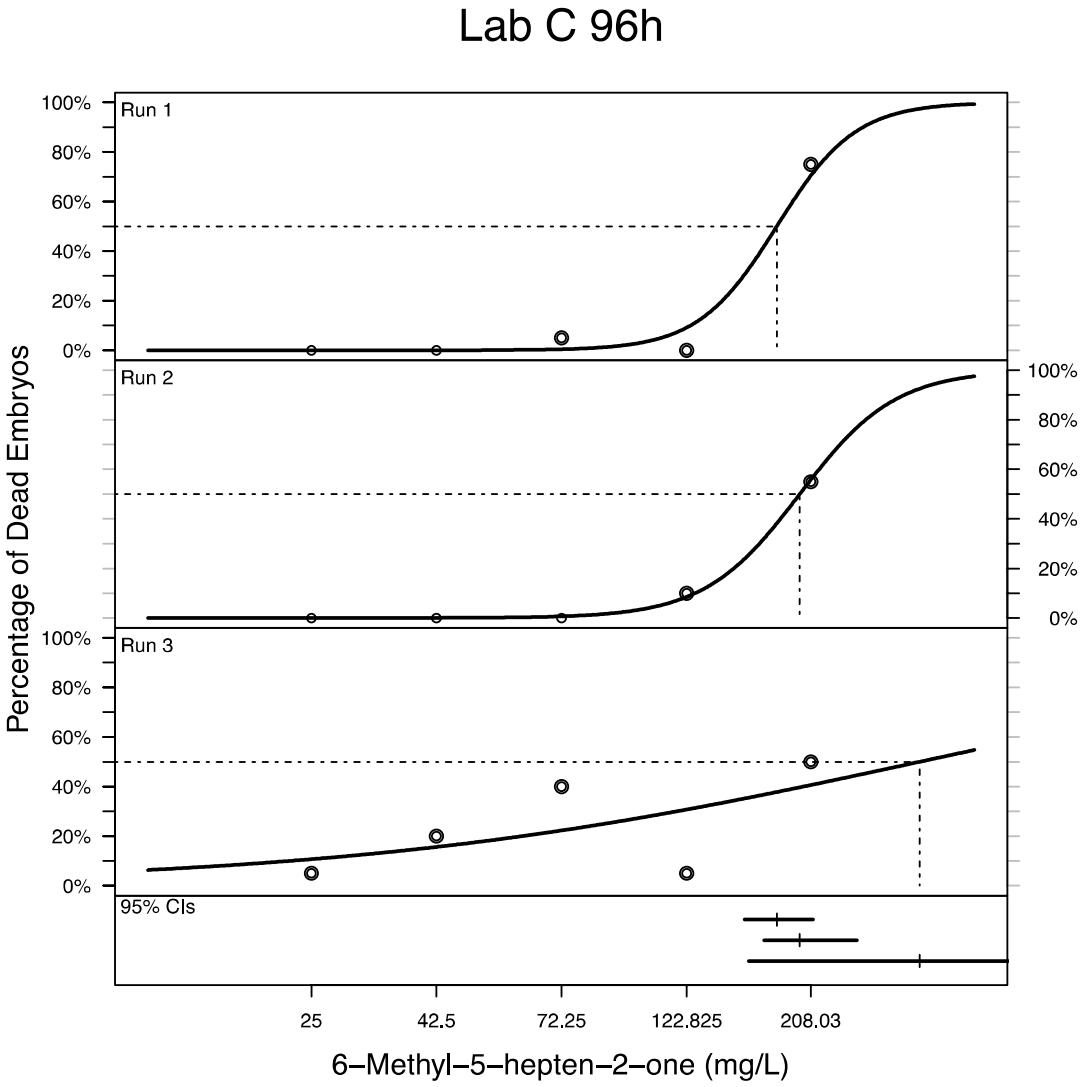

Lab G 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
8
12
18
27
40.5
2,3,6–Trimethylphenol (mg/L)

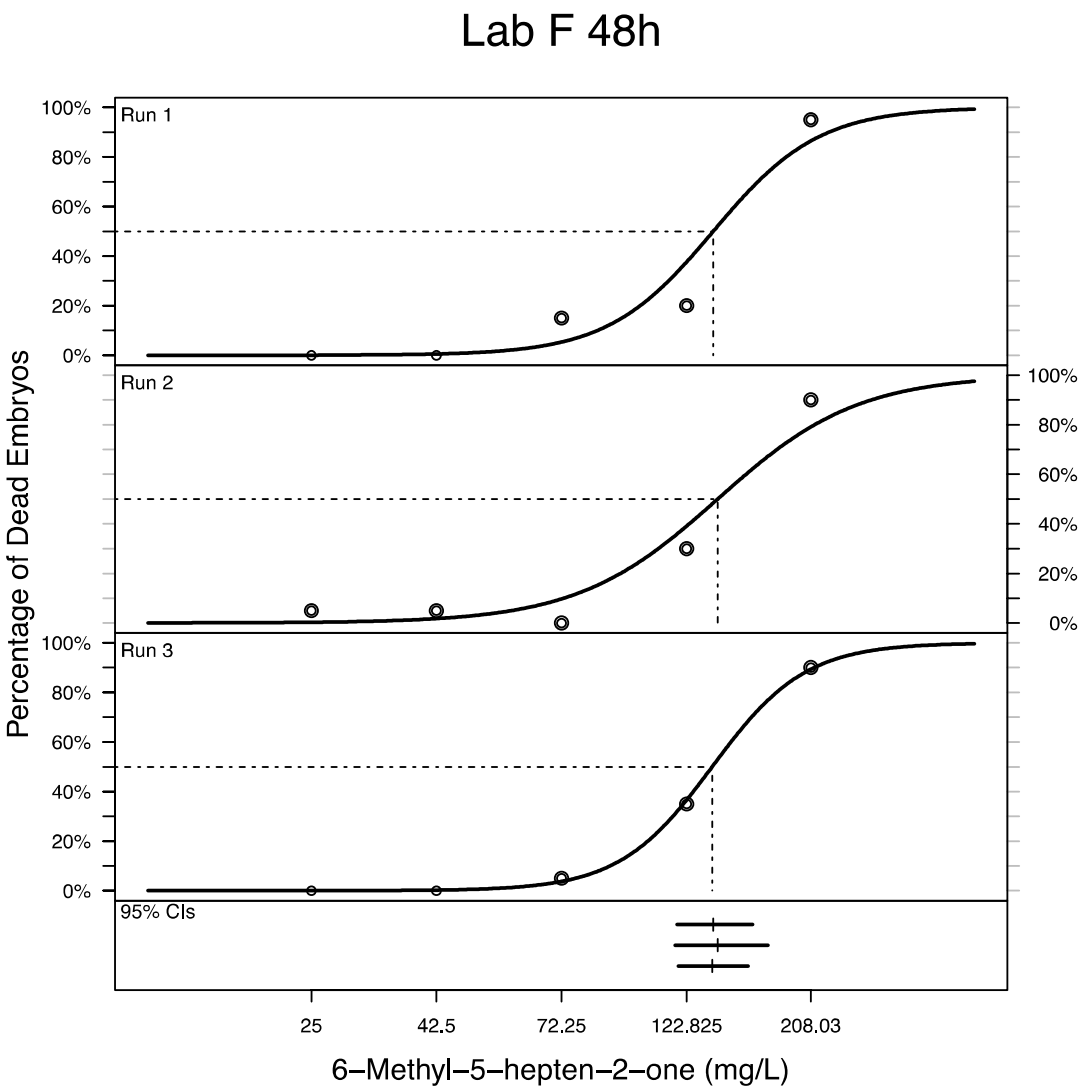
6-Methyl-5-hepten-2-one

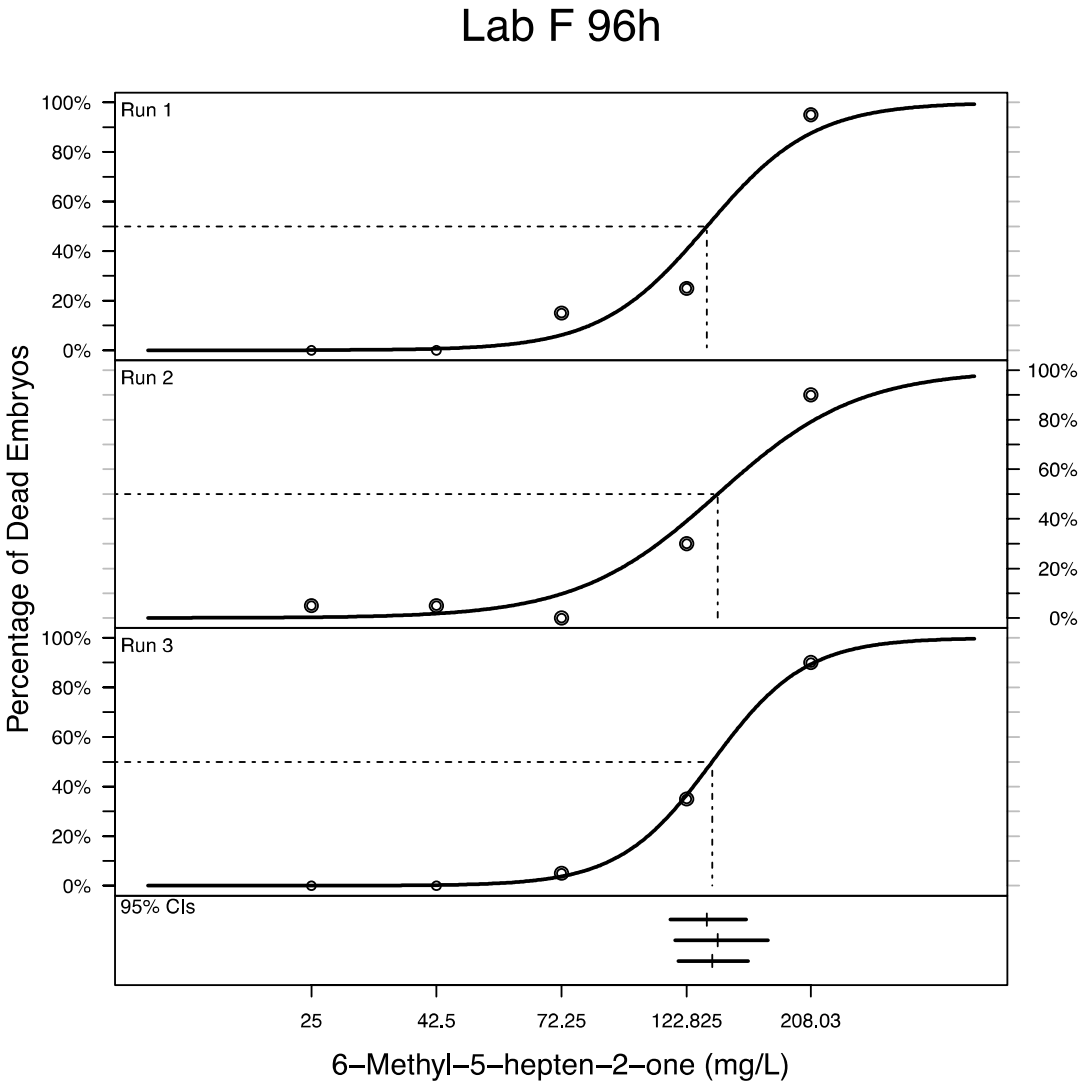



Lab B 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
25
42.5
72.25
122.825
208.03
6–Methyl–5–hepten–2–one (mg/L)


Lab C 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
25
42.5
72.25
122.825
208.03
6–Methyl–5–hepten–2–one (mg/L)


Lab C 96h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
25
42.5
72.25
122.825
208.03
6–Methyl–5–hepten–2–one (mg/L)


Lab F 48h
Run 1
Run 2
Run 3
95% CIs
100%
80%
60%
40%
20%
0%
Percentage of Dead Embryos
25
42.5
72.25
122.825
208.03
6–Methyl–5–hepten–2–one (mg/L)


Lab F 96h
Run 1
Run 2
Run 3
95% CIs
100%
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60%
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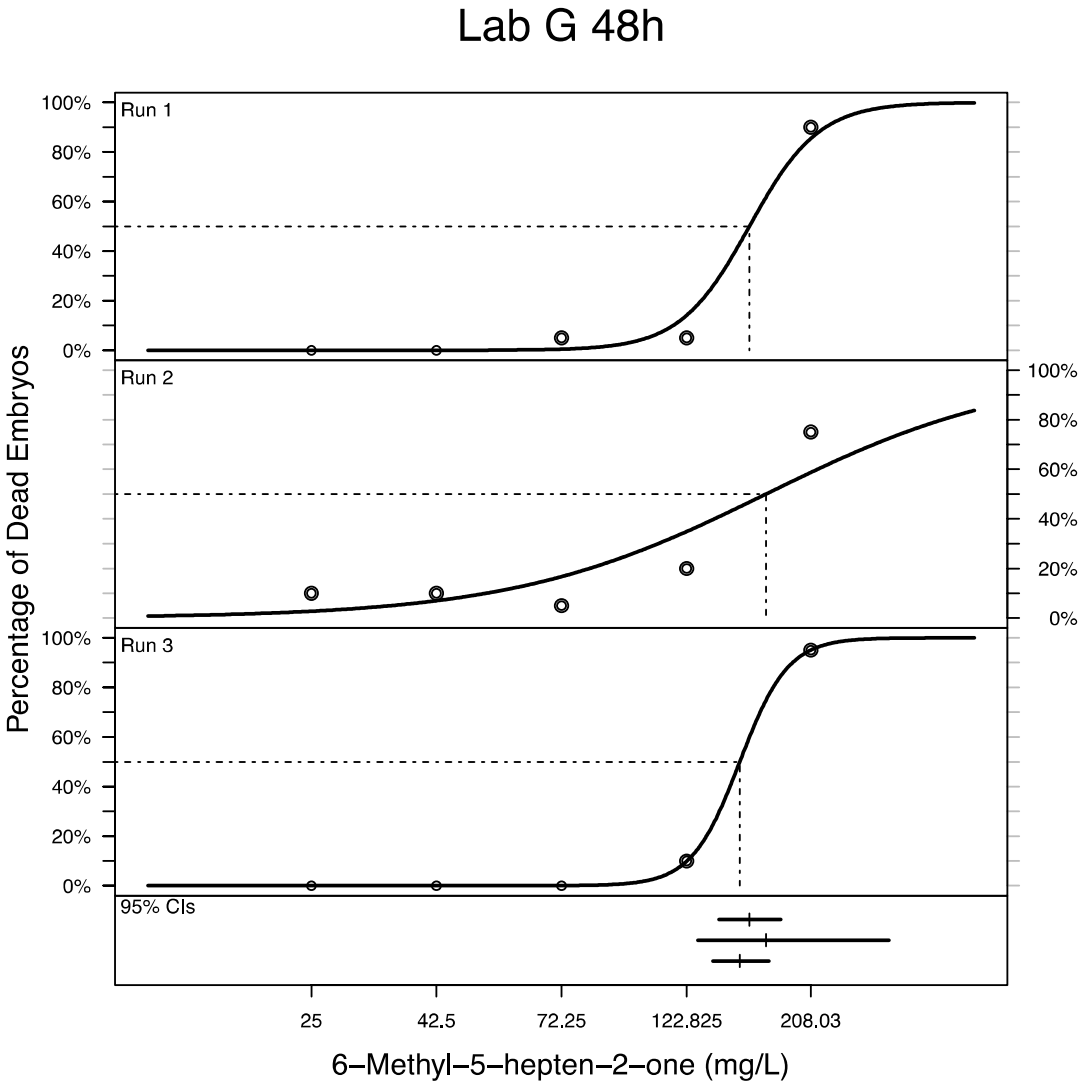
Percentage of Dead Embryos
25
42.5
72.25
122.825
208.03
6–Methyl–5–hepten–2–one (mg/L)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Percentage of Dead Embryos
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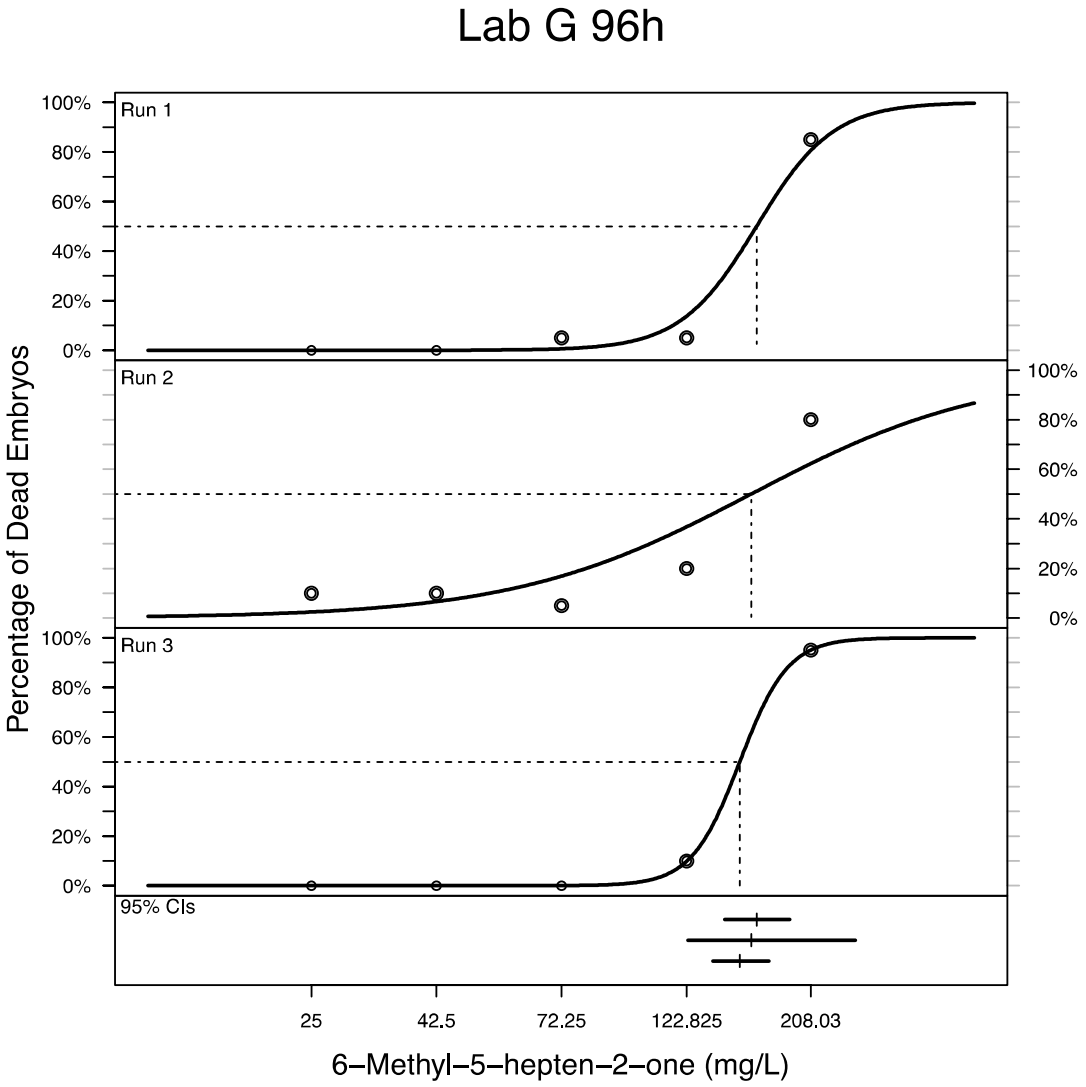
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Percentage of Dead Embryos
25
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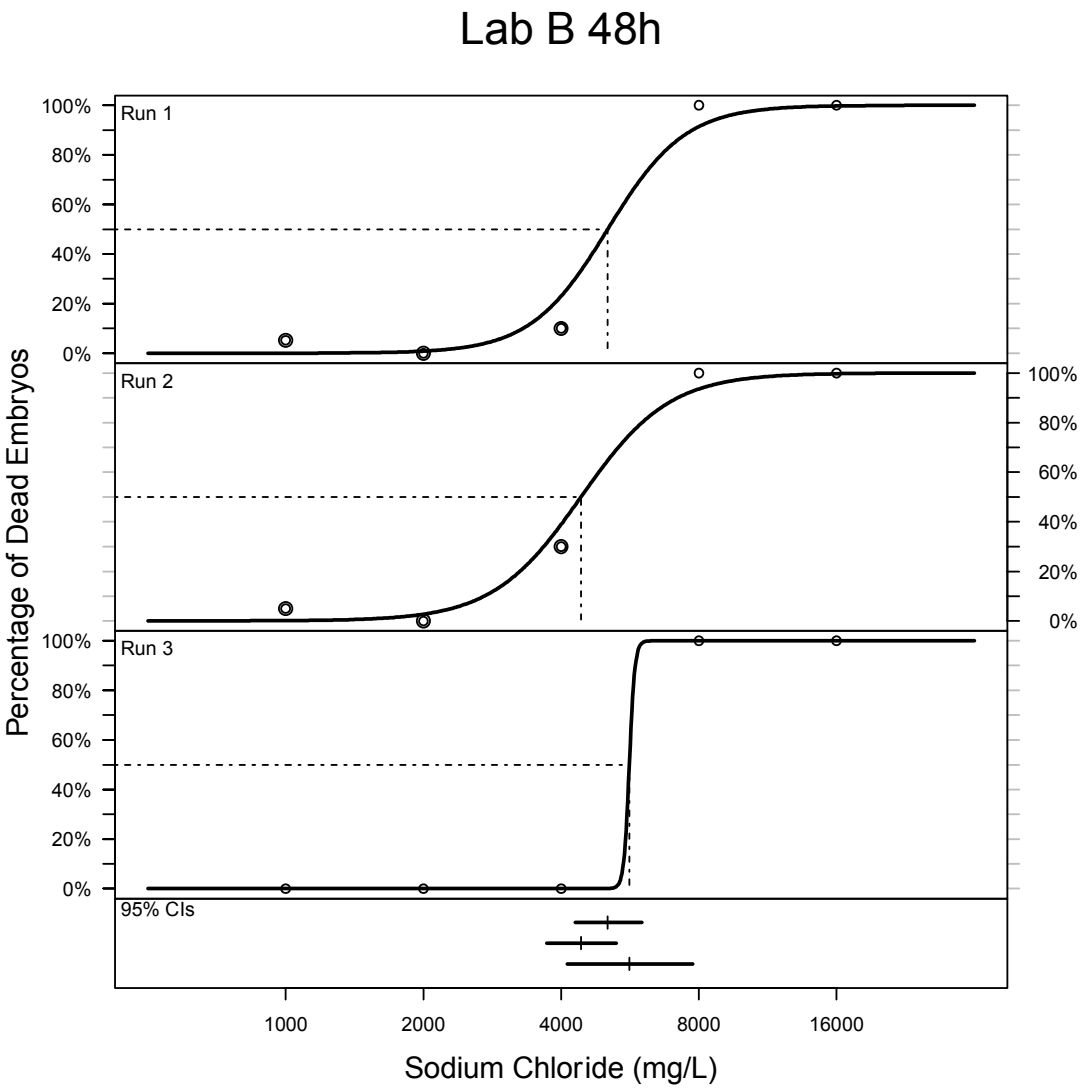
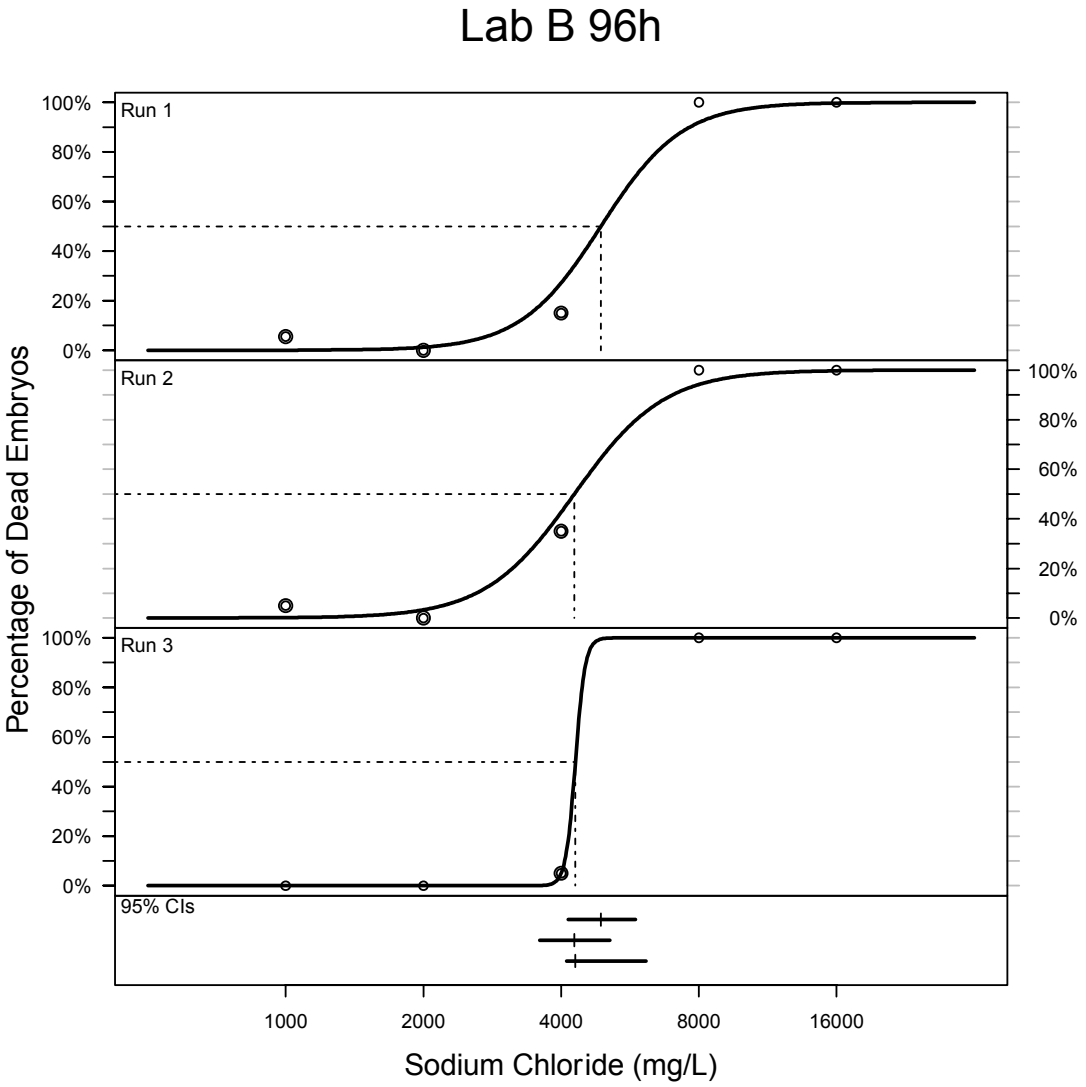
6–Methyl–5–hepten–2–one (mg/L)

Sodium Chloride

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Run 3
95% CIs
100%
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60%
40%
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0%
Percentage of Dead Embryos
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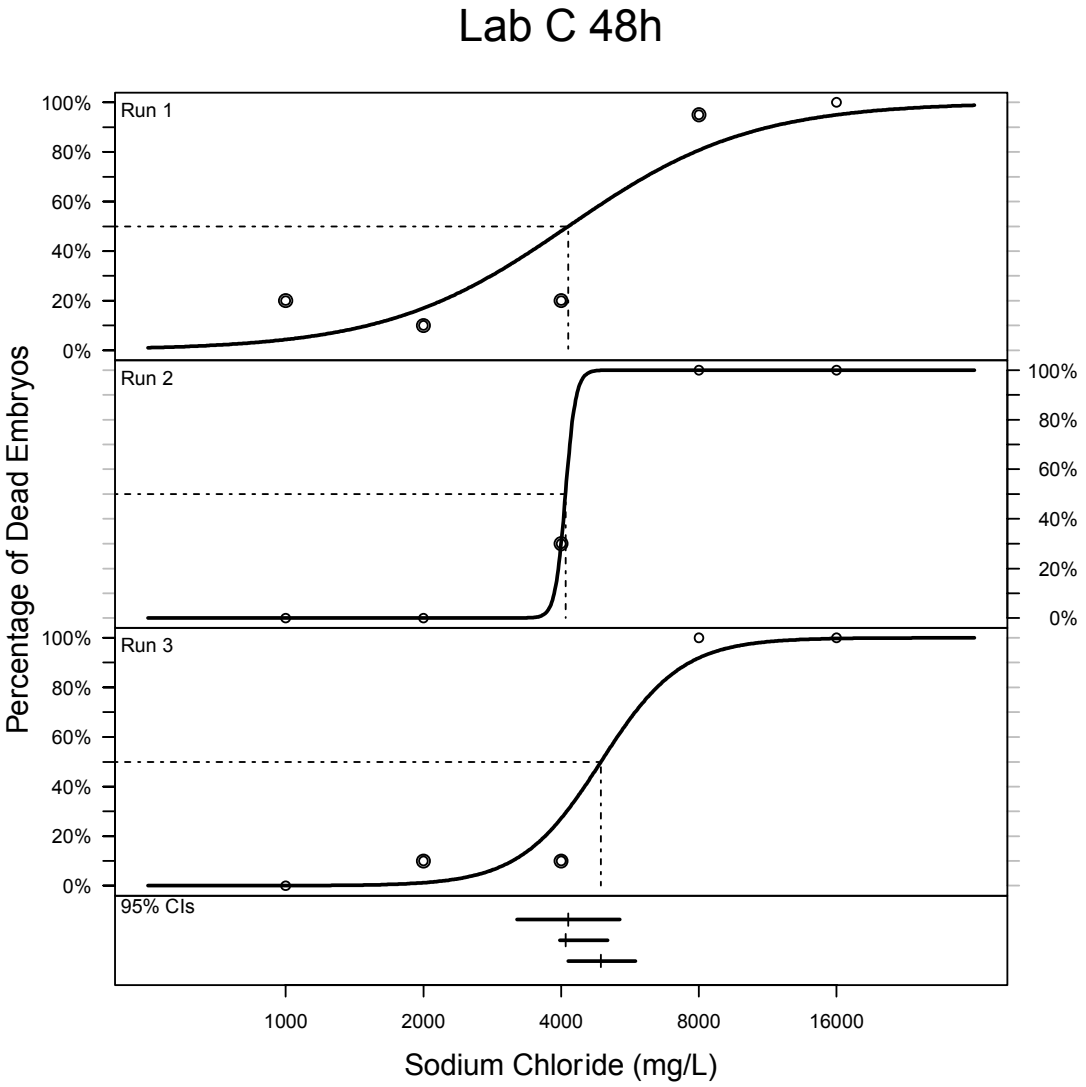
8000
16000
Sodium Chloride (mg/L)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Run 3
95% CIs
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Percentage of Dead Embryos
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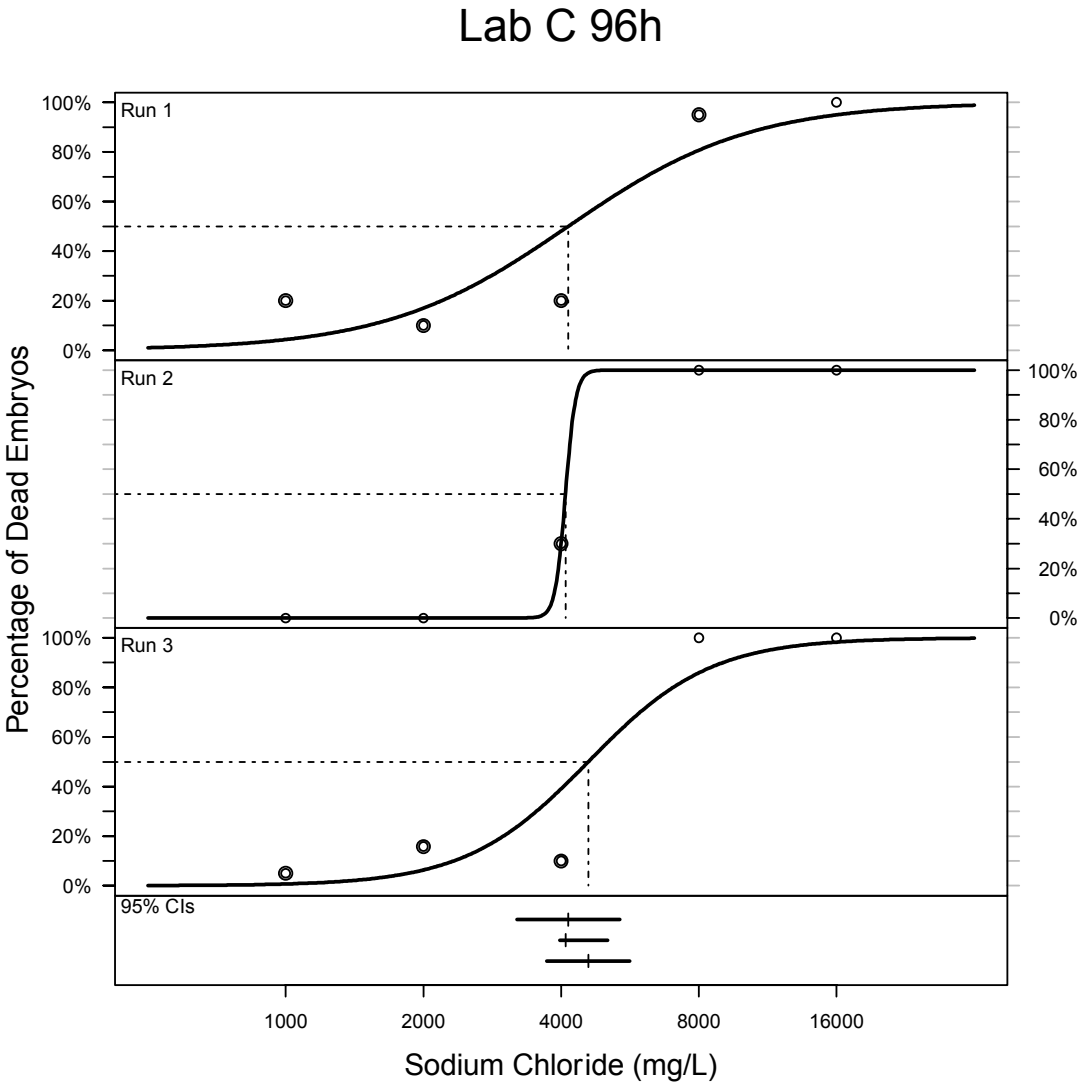
Sodium Chloride (mg/L)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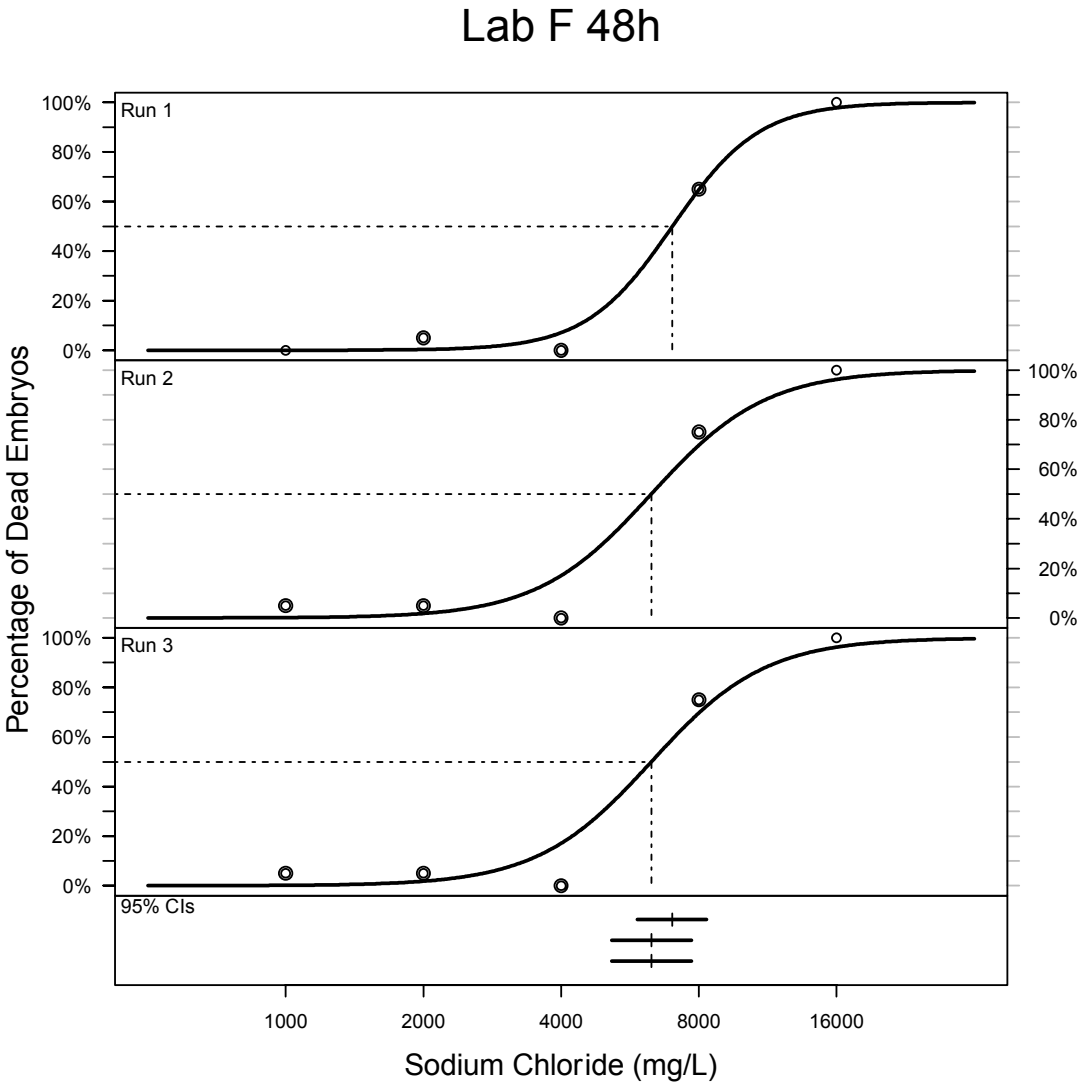
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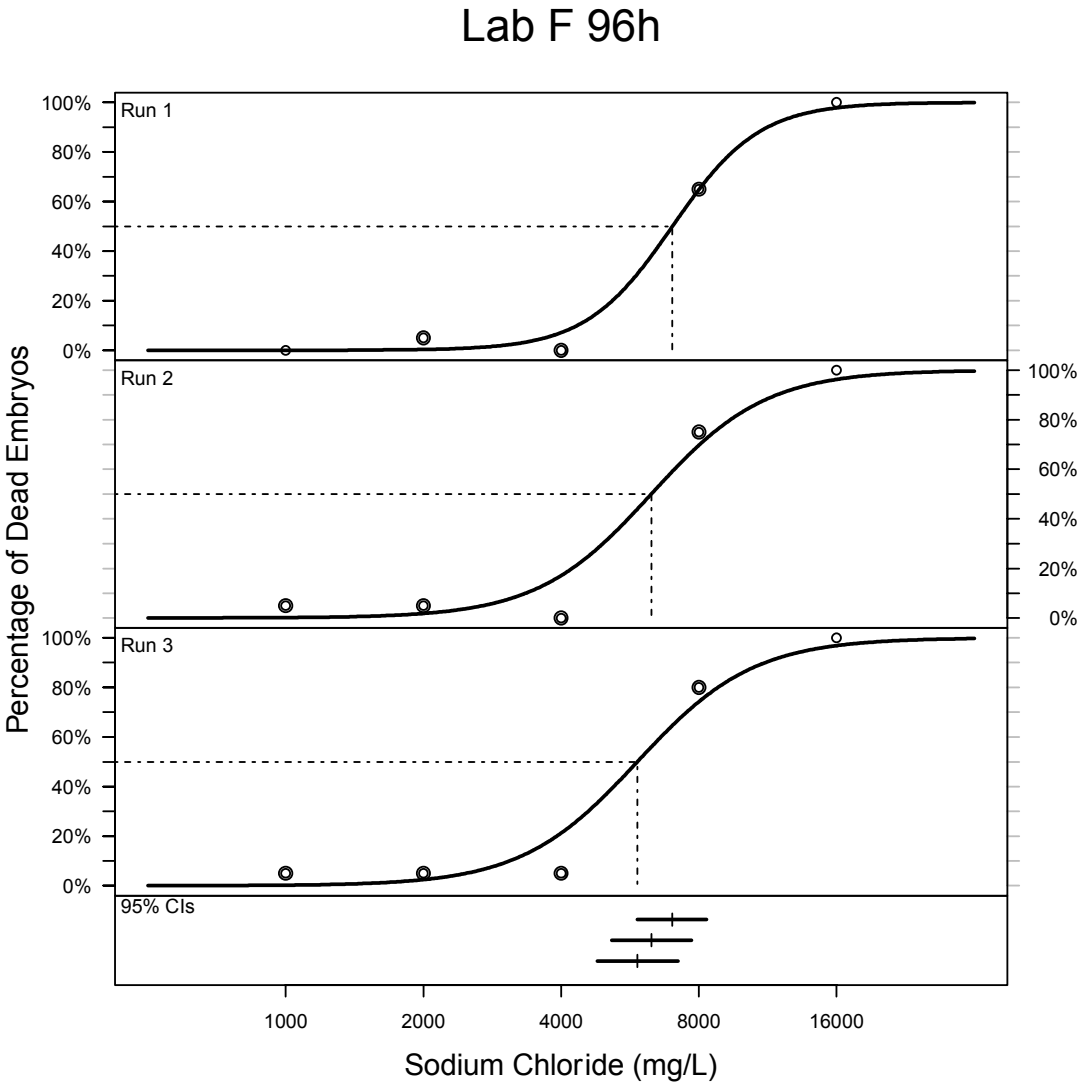
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Percentage of Dead Embryos
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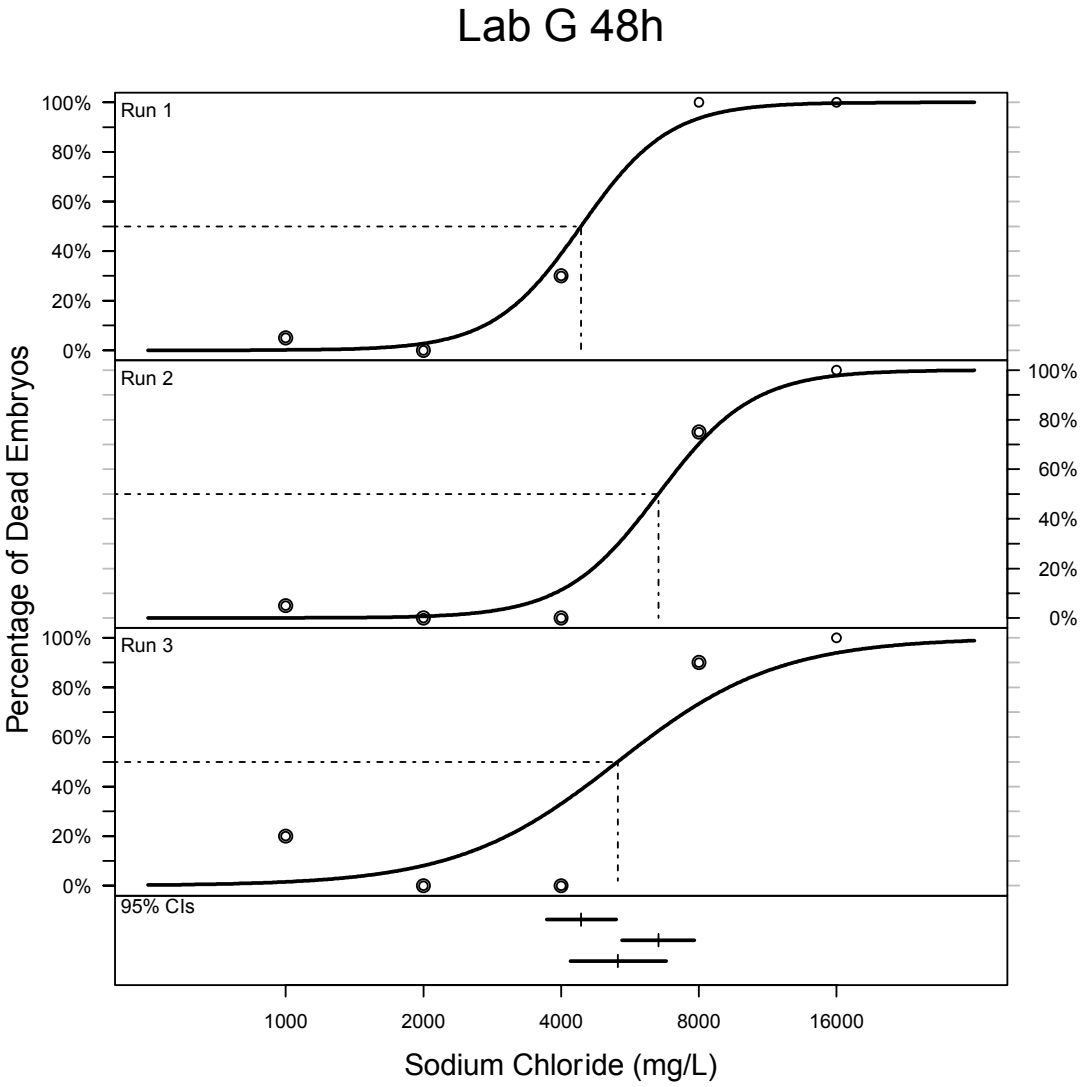
16000
Sodium Chloride (mg/L)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95% CIs
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Percentage of Dead Embryos
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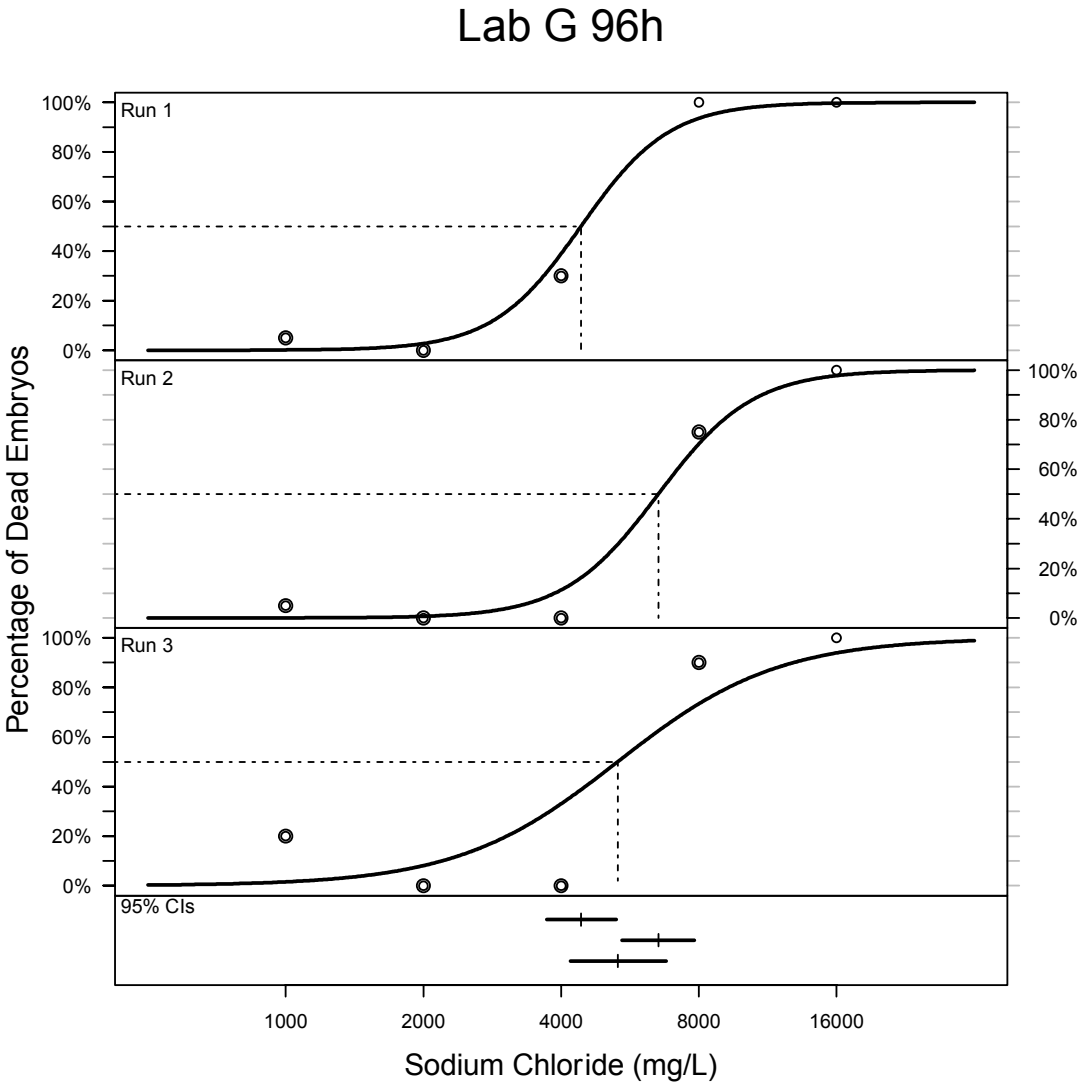
Sodium Chloride (mg/L)


Lab G 96h
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95% CIs
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Percentage of Dead Embryos
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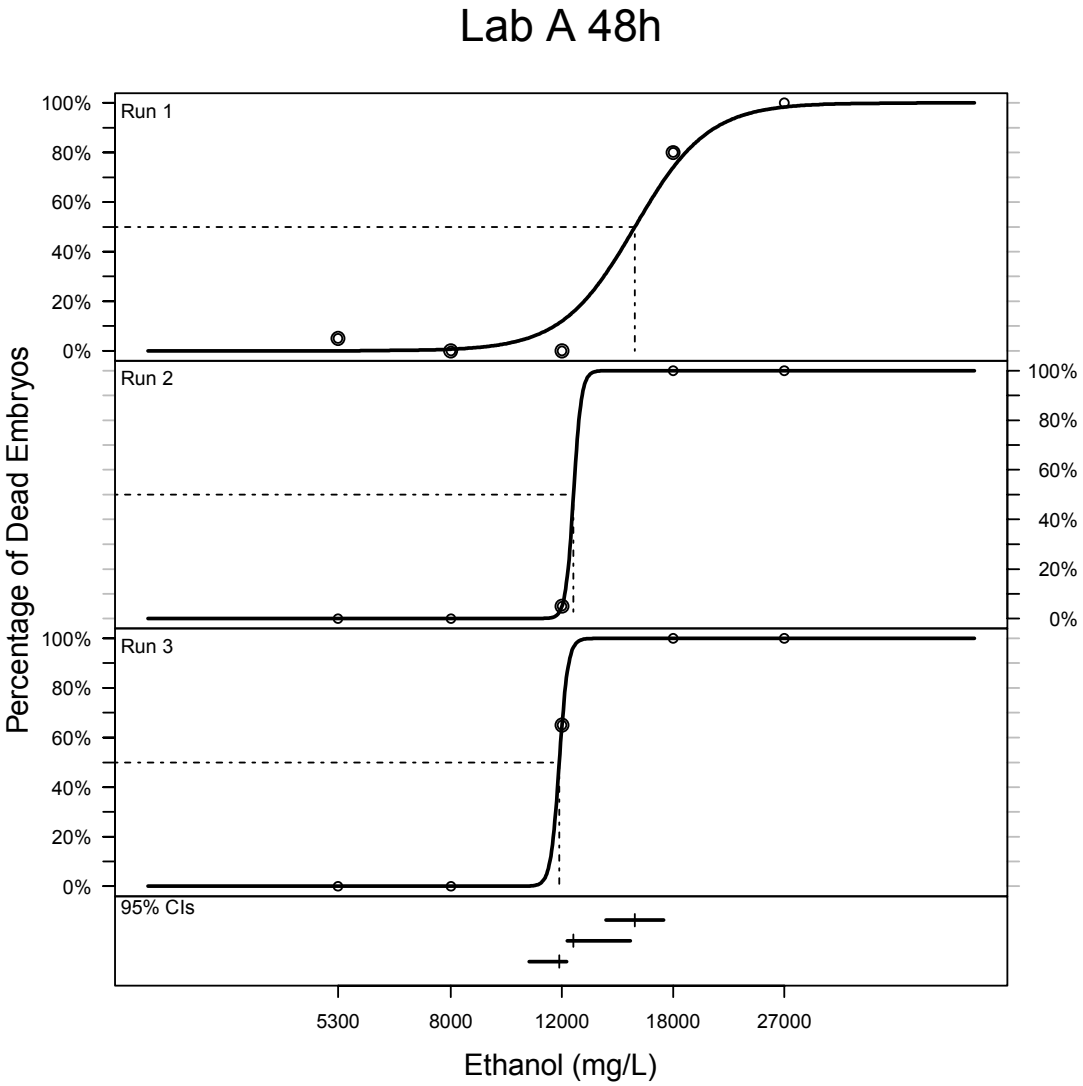
Sodium Chloride (mg/L)

Ethanol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Run 1
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Run 3
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Percentage of Dead Embryos
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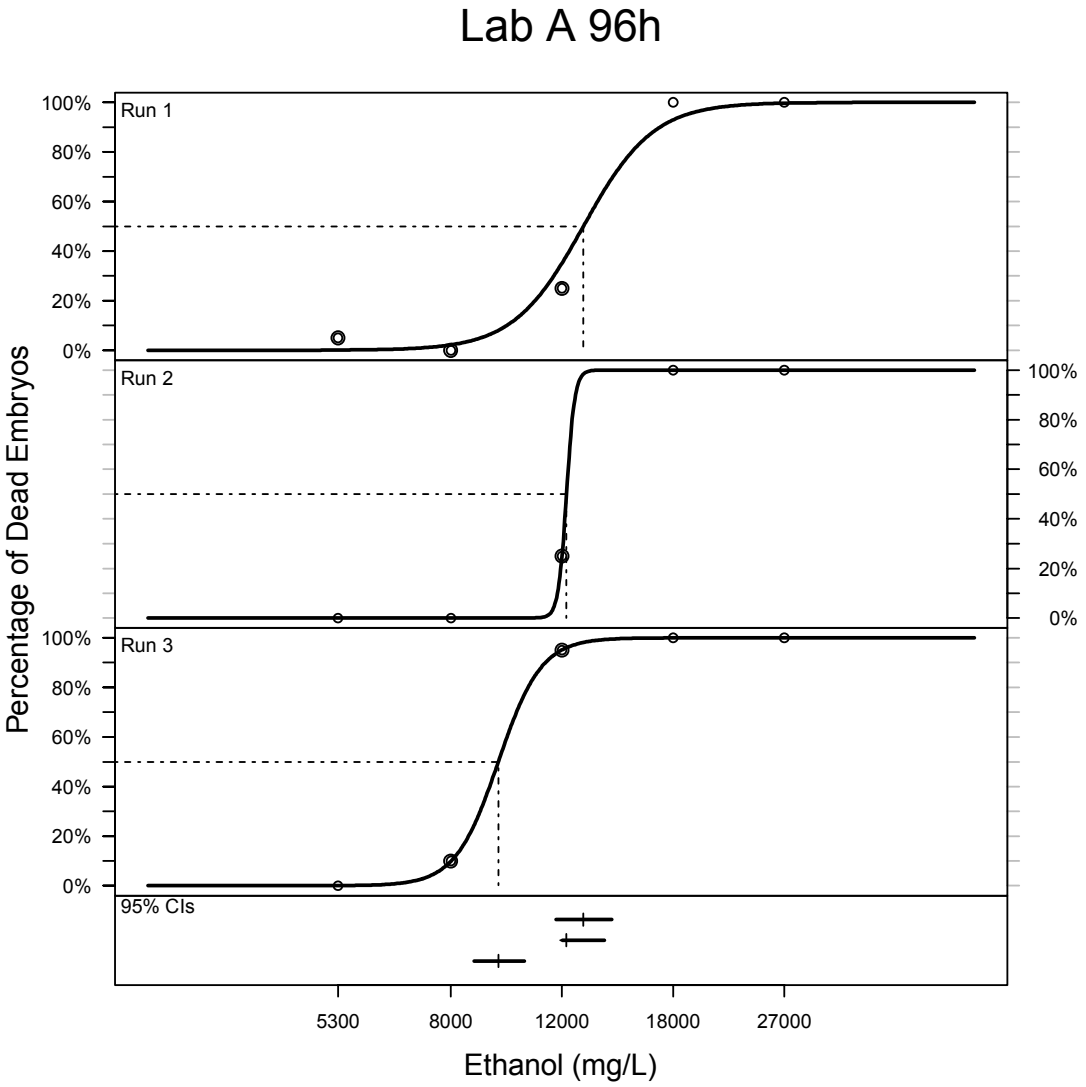
Ethanol (mg/L)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Percentage of Dead Embryos
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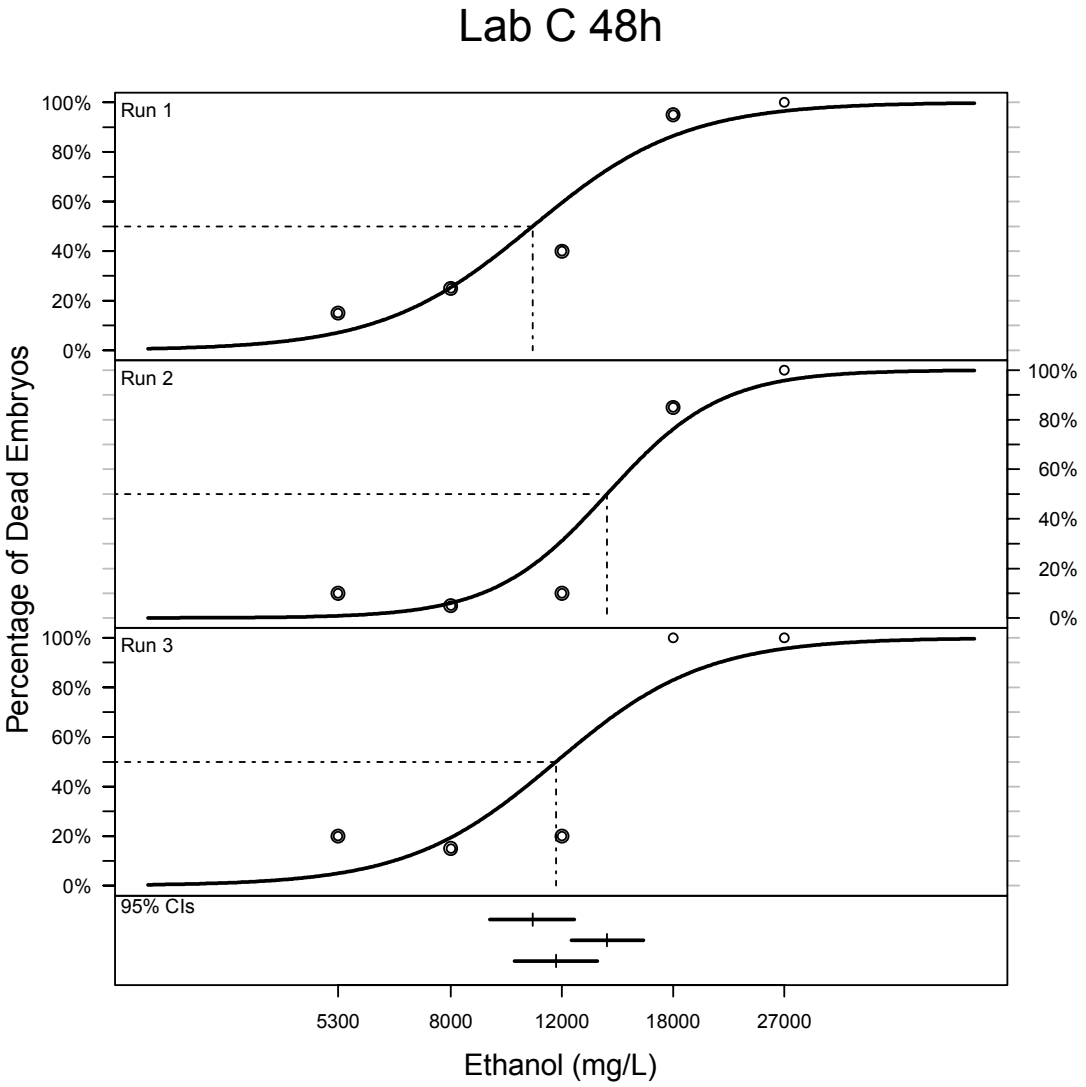
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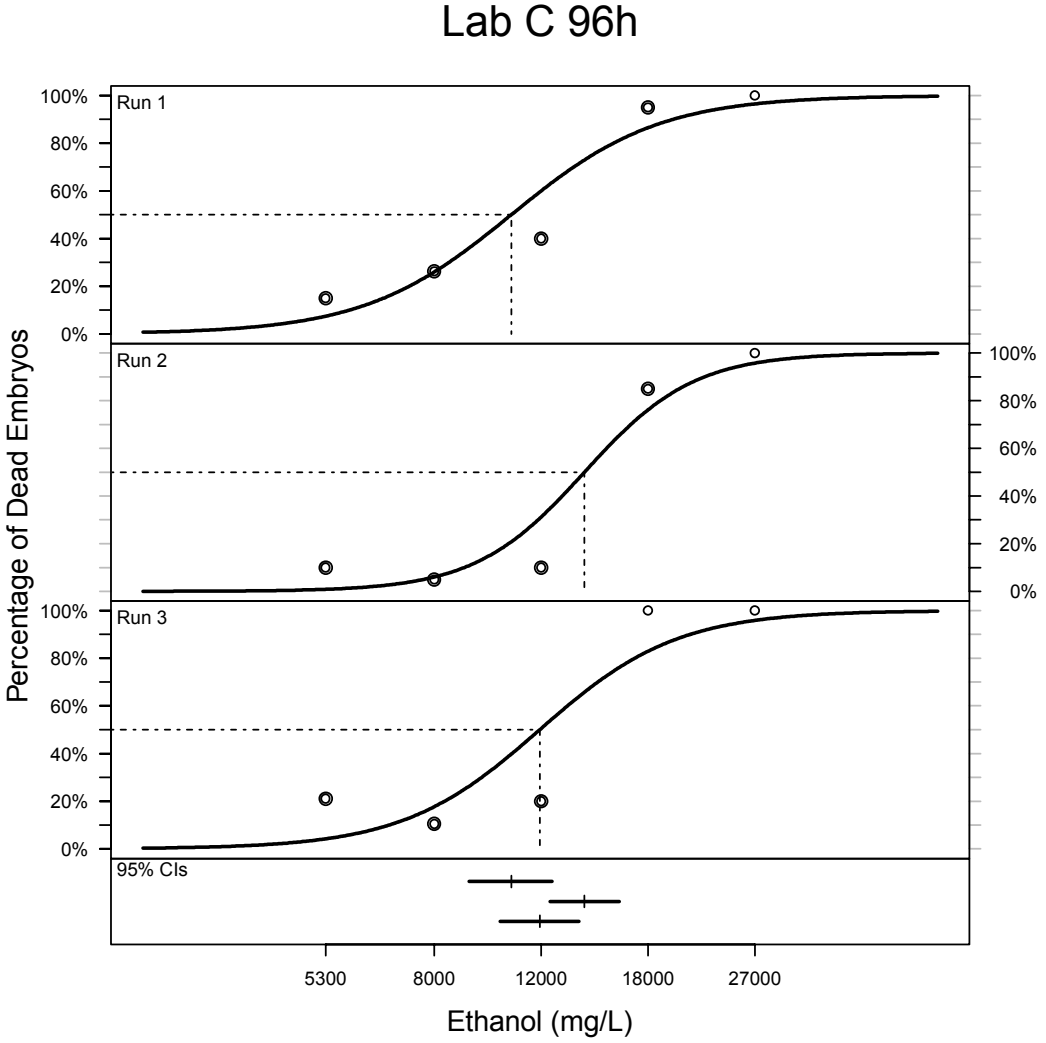
Ethanol (mg/L)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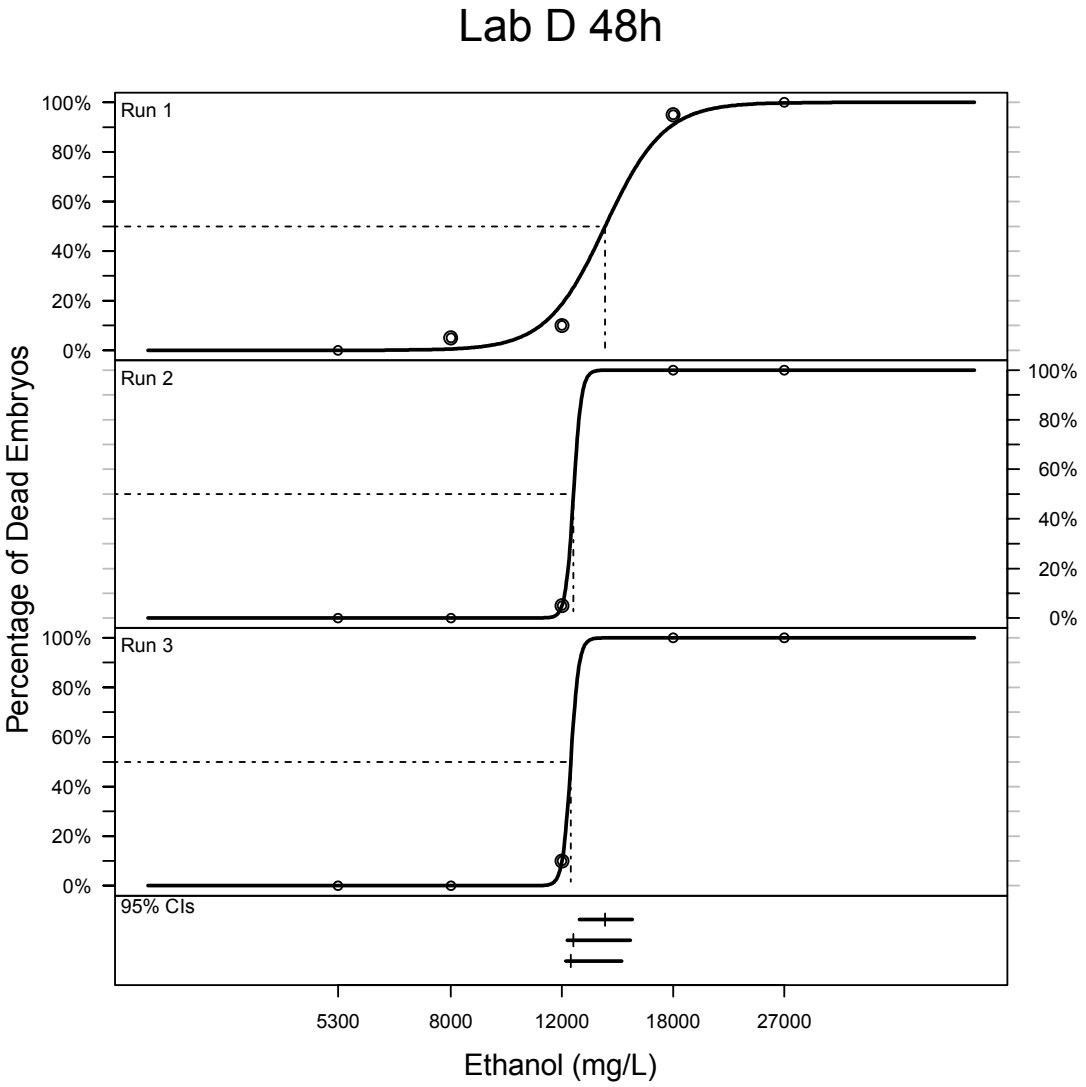
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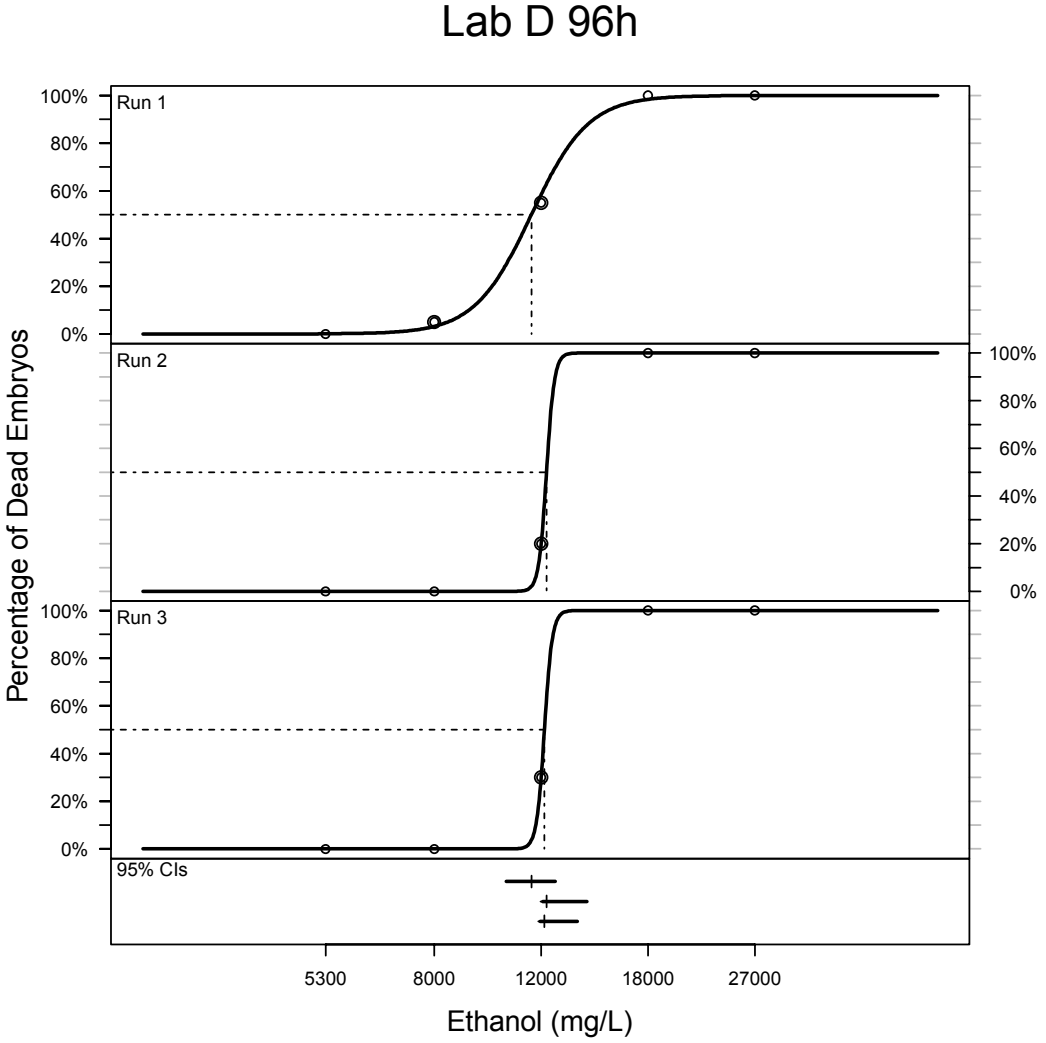
Ethanol (mg/L)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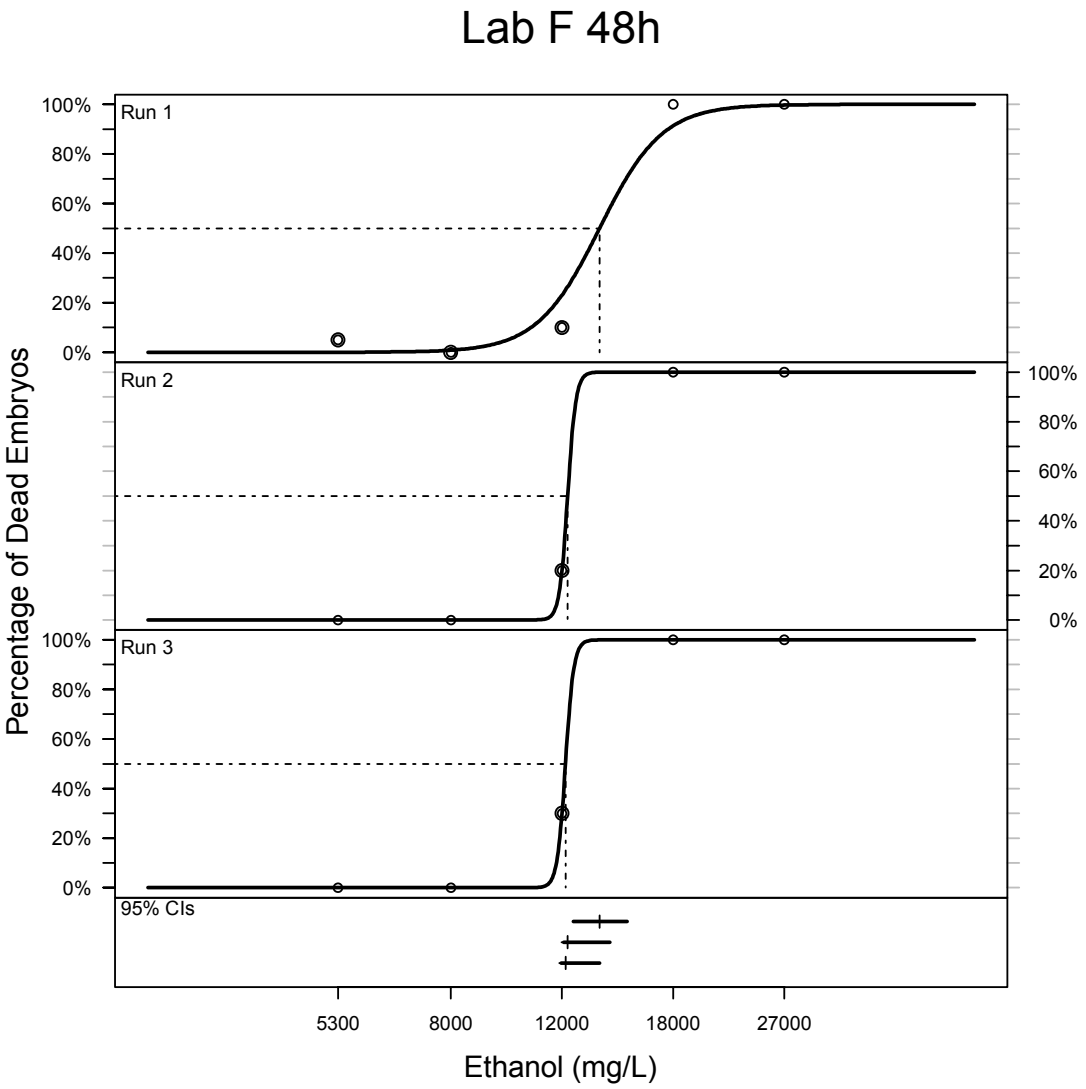
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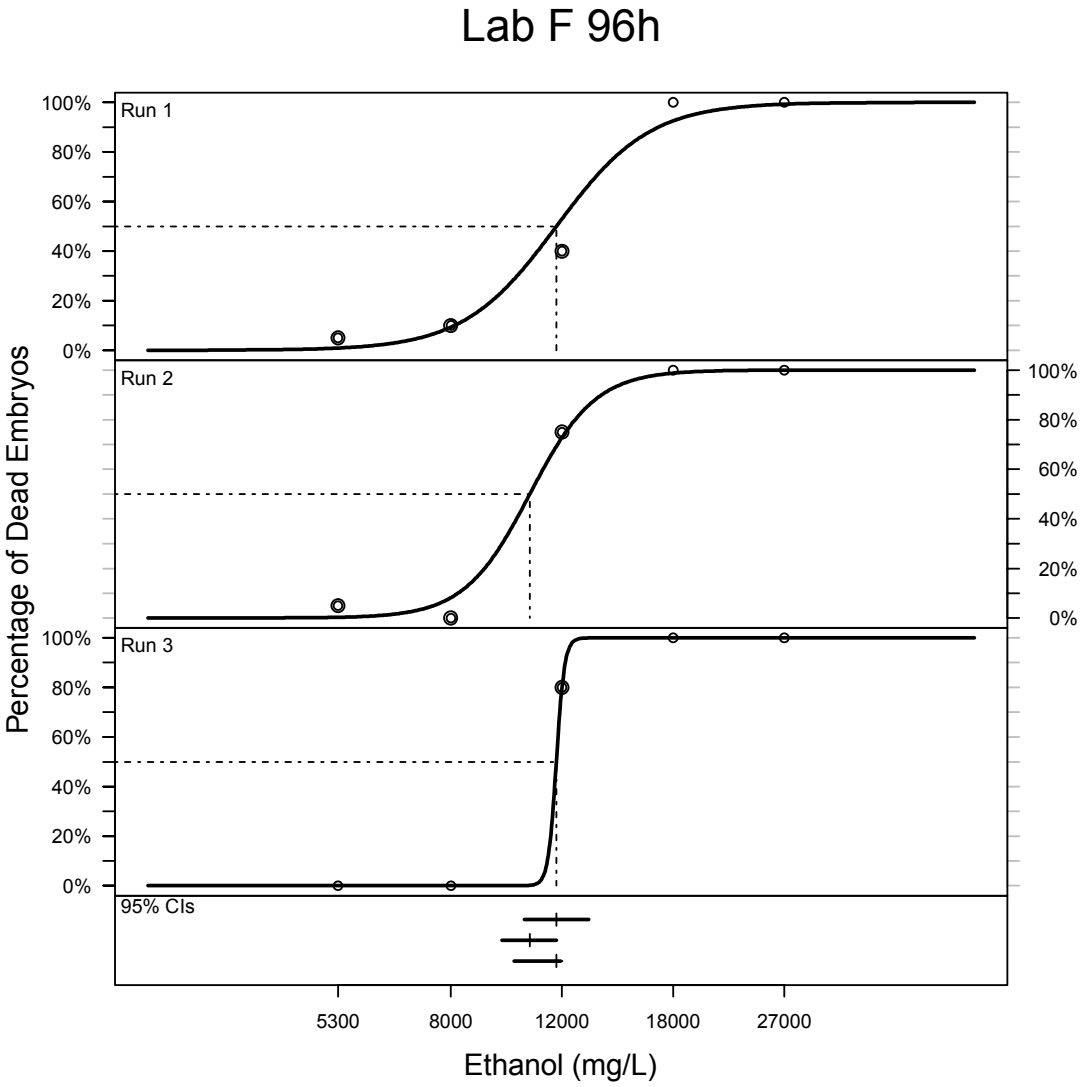
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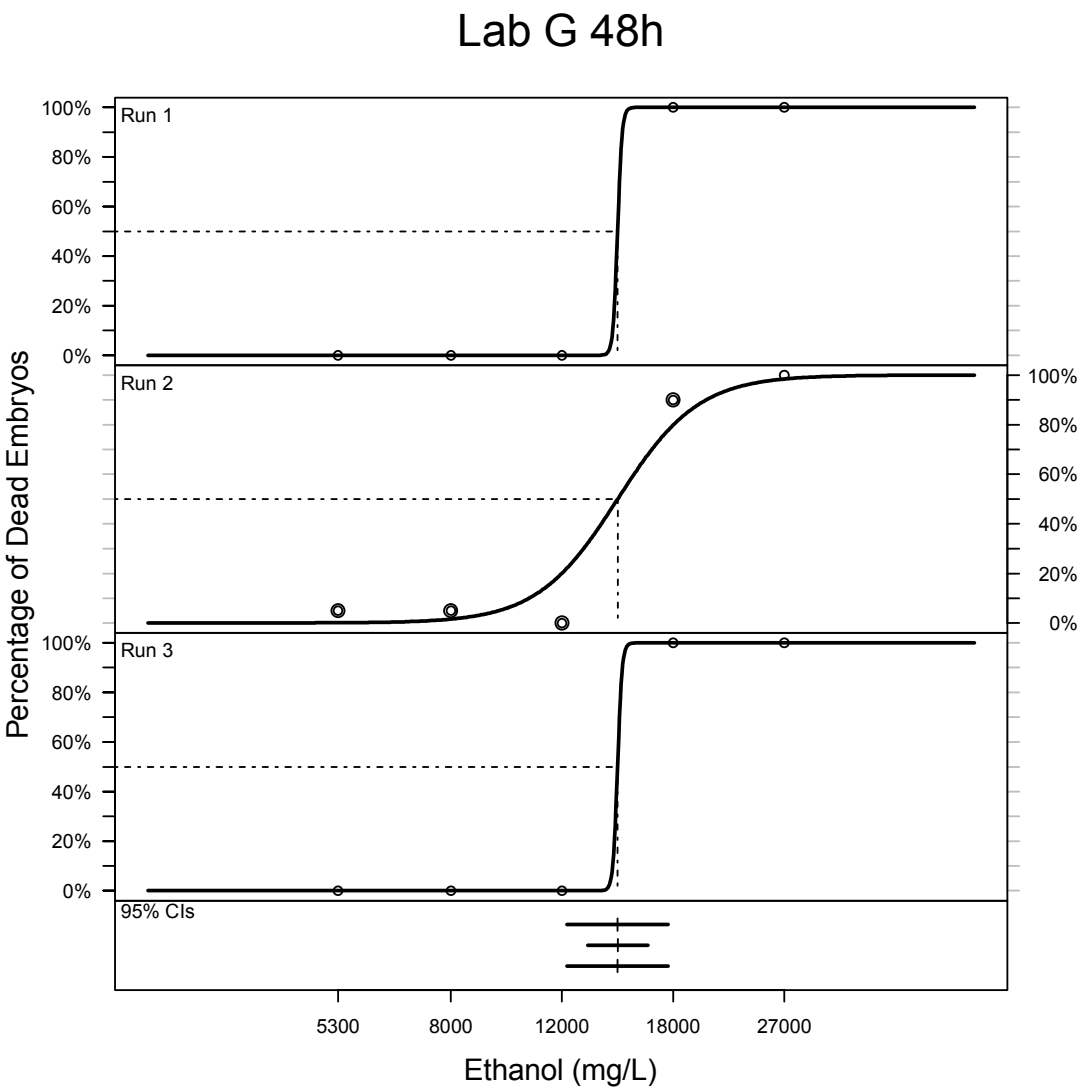
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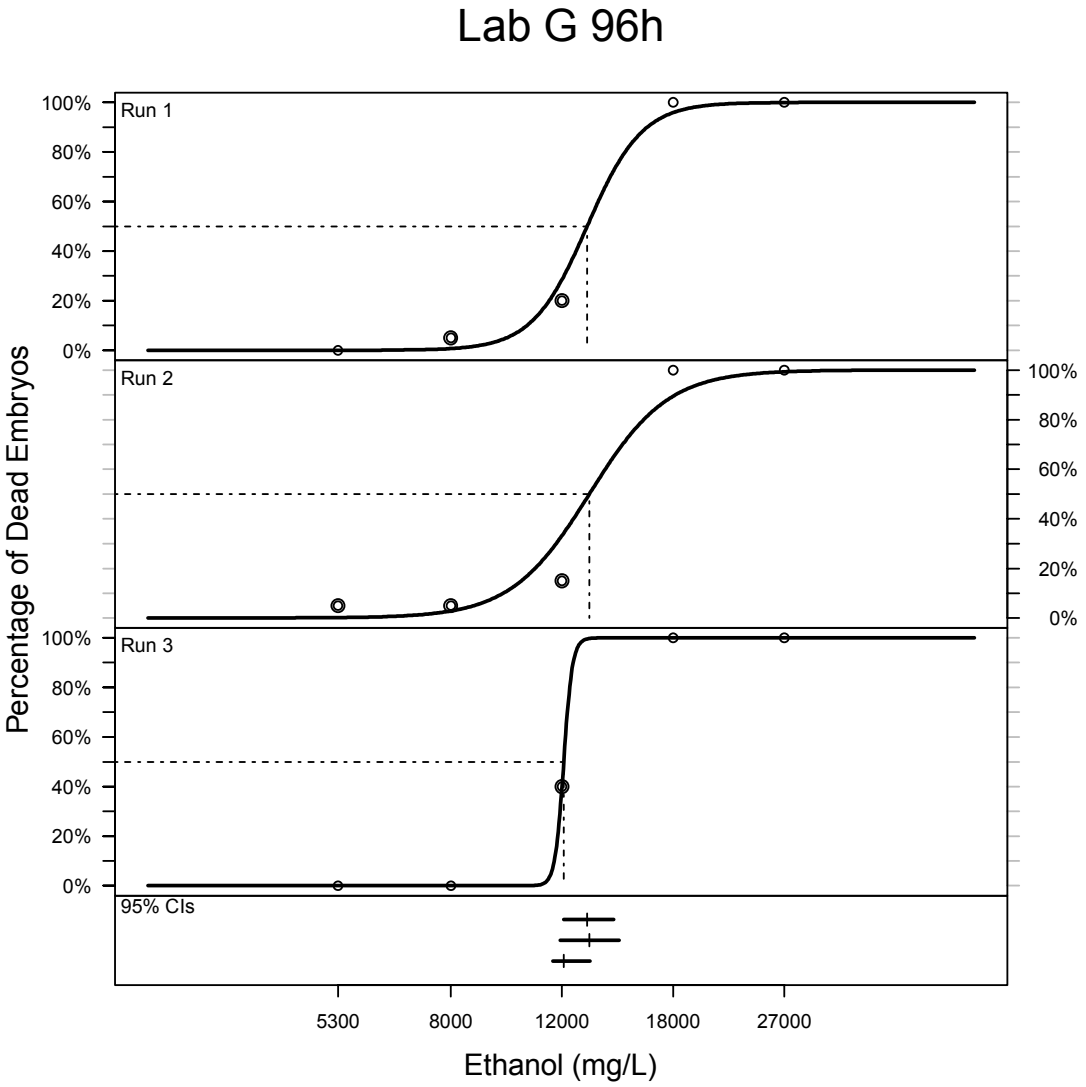
Ethanol (mg/L)


Lab G 96h
Run 1
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95% CIs
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Percentage of Dead Embryos
5300
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12000
18000
27000
Ethanol (mg/L)

Zebrafish Embryo Toxicity Test

Evaluation of transferability, intra- and interlaboratory reproducibility

Trial Plan for Phase 1a – Transferability

TP_ZFET_OECD_1a_V01.7

June 17th 2009

Title	**Zebrafish Embryo Toxicity Test: Evaluation of transferability, intra- and interlaboratory reproducibility – Phase 1a: Transferability**		
Sponsor:	**-**		
Identification:	*TP_ZFET_OECD_1a*		
Start Date:	**28 April 2009**	**End Date:**	

Trial Coordinator	Name	Marlies Halder
	Date	January 2009
	Signature	
Reviewer 1	Name	Participating laboratories
	Date	27 March 2009
	Signature	
Reviewer 2	Name	Validation Management Group
	Date	28 April 2009
	Signature	

PAGES OF CHANGES

Date of change	Version number	Changed pages/sections	Summary of the change(s)	Changed by/sign
12/06/2009	V01.7	6, 8 and 11	Minor editorial changes	Marlies Halder on behalf the VMG
		7	6: Time schedule and design of the study. See revised details of step 6	Marlies Halder on behalf the VMG
		9,10	7: Study performance 4 subsections were added. Details on presaturation, daily renewals and measurements on test conditions were described	Marlies Halder on behalf the VMG
		10	8.2 pH adjustment amended	Marlies Halder on behalf the VMG
		11	8.3 Definition of fresh test concentration; temperature of dilution water was added	Marlies Halder on behalf the VMG
		Annex 3	Shipment instructions; Notification of Scott Belanger	Marlies Halder on behalf the VMG

Table of Contents

Zebrafish Embryo Toxicity Test
Evaluation of transferability, intra- and interlaboratory reproducibility

Phase 1a: Transferability

1. Introduction

The acute fish toxicity test is a mandatory component in the environmental safety assessment of industrial chemicals, agrochemicals, pharmaceuticals, feed stuff etc. In the European Union, Council Directive 86/609/EEC on the protection of laboratory animals (EC, 1986) and, in particular, the legislation on chemicals (REACH; EC 2007) demand that tests on vertebrate animals are reduced, refined or replaced whenever possible.

One of the most promising alternative approaches to the LC50 96h fish toxicity test (OECD 203 [OECD, 1992]; C.1 [EC, 2008]) is based on the use of fish embryos.

In Germany, the Fish Egg Toxicity test (DIN 2001) was validated and replaced the 48 h acute fish test for routine whole effluent testing in 2005. Recently, a modified international version of the fish egg toxicity test was published (ISO 2007).

Extensive efforts have been undertaken to adapt the method to also meet chemical testing requirements (Nagel 2002, Braunbeck *et al.,* 2005, Lammer *et al.,* 2009). In fall 2005, the German Federal Environment Agency submitted the draft guideline “Fish embryo toxicity (FET) test” to the OECD Test Guideline program 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 teleconference 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 demonstrates that the zebrafish fish embryo test correlates well with acute fish toxicity tests (Lammer *et al.* 2009).

The ad hoc Expert Group on the Fish Embryo Toxicity Test noted that most data are available for the zebrafish embryo toxicity test, however, data providing sufficient evidence for the reproducibility of the method are lacking.

2. Purpose of the study

The zebrafish embryo toxicity test (ZFET) is designed to determine the lethal effects of chemicals on embryonic stages of fish and constitutes an alternative test method to the acute toxicity tests with juvenile and adult fish, i.e. the OECD Test Guideline 203 (OECD 1992).

Following the advice of the OECD ad hoc Expert Group on Fish Embryo Tests, OECD decided to perform a ring trial in a restricted number of laboratories. The purpose is to evaluate:

- the transferability,
- the intralaboratory reproducibility, and
- the interlaboratory reproducibility of the ZFET.

The study is steered by a validation management group.

The study is divided into two phases, where Phase 1 constitutes the transferability of the ZFET from the Lead laboratory to the other laboratories (Phase 1a – Transferability/Training) and consequent the testing of five substances (Phase 1b). Based on the outcome of Phases 1a and 1b, the standard operation procedure (SOP) might undergo revisions. In Phase 2, a larger set of substances will be tested.

3. Validation management group

The validation management group (VMG) will steer the study and is responsible for the overall study design. Specific roles and responsibilities are listed below:

Name	**Affiliation/contact**	**Role**
Marlies Halder François Busquet	JRC/IHCP/IVM-ECVAM marlies.halder@jrc.ec.europa.eu francois.busquet@jrc.ec.europa.eu	Coordination/reporting
André Kleensang	JRC/IHCP/IVM-ECVAM andre.kleensang@jrc.ec.europa.eu	Data analysis
Patric Amcoff	OECD patric.amcoff@oecd.org	OECD TG Program
Thomas Braunbeck	University of Heidelberg braunbeck@zoo.uni-heidelberg.de	Lead laboratory, SOP; UBA representative
Scott Belanger	Procter & Gamble belanger.se@pg.com	Chemical analysis, participating laboratory
Adam Lillicrap	NIVA Adam.Lillicrap@niva.no	Independent adviser

4. Participating laboratories

Name	responsible/contact	Role
University of Heidelberg	Thomas Braunbeck braunbeck@zoo.uni-heidelberg.de	Lead laboratory
Procter & Gamble	Scott Belanger belanger.se@pg.com	Participating laboratory
Ipo-Pszczyna	Przemysław Fochtman fochtman@ipo-pszczyna.pl	Participating laboratory
IVM	Juliette Legler juliette.legler@ivm.vu.nl	Participating laboratory
UFZ	Stefan Scholz Stefan.Scholz@ufz.de	Participating laboratory
RIVM	Leo van der Ven Leo.van.der.Ven@rivm.nl	Participating laboratory
VITO	Hilda Witters hilda.witters@vito.be	Participating laboratory

Full contact details and alternate person to be contacted are given in Annex 3.

5. Standard operation procedure

The use of the SOP_ZFET_OECD_V02.8 is mandatory. Any deviation from the SOP must be reported in the reporting template.

6. Time schedule and design of the study

The study design covers training / transferability aspects of the method and allows to intervening at any stage.

Week	Step	Action	Responsible
		Distribution of 3,4-dichloroaniline – labs confirm receipt	Thomas Braunbeck
		Discussion of SOP & Trial plan with participating labs – labs send comments to lead lab and ECVAM – Teleconference call on **27/03/2009 15-17h00 (CET)**	Lead laboratory, ECVAM and participating labs
		If necessary, revision of SOP, Trial Plan, Reporting template and repetition of step above	VMG
		P&G will contact the labs to prepare documents necessary for shipment of samples	Scott Belanger
0	1	Distribution of final Phase 1a trial plan, SOP, reporting templates via e-mail – labs confirm receipt	ECVAM
1	2	a) Preparation of 3,4DCA stock solution (see 8.2) and sending to P&G for analysis b) Testing of 3,4-dichloroaniline (6 concentrations) in 1 run; c) Submission of data to ECVAM	Participating labs
2	3	Analysis of data	ECVAM
2	4	Discussion of data	VMG
2	5	If necessary, revision of SOP and repetition of step 1, 2b & c	Lead laboratory, VMG
3	**6.1**	**If you have not yet sent samples of the stock solution (see step 2) to P&G for analysis or if you need to prepare a new stock solution, please send samples as described in Annex 3 to Scott Belanger**	**Participating labs**
3	**6.2**	**a) Use stock solution prepared under step 2 for the below 3 independent runs** **b) Testing of 3,4-dichloraniline (6**	**Participating labs**

		concentrations) in 3 independent runs ***("independent" means that the experiments are performed with different batches of zebrafish eggs, on different days and with newly prepared test concentrations)*** **c) Submission of data to ECVAM**	
7	7	Analysis of data	ECVAM
1 - 8	8	Analytical measurements of stock solutions and P&G samples	P&G
9	9	Discussion of data & decision on progression to Phase 1b	VMG

7. Study performance

7.1. General considerations

The zebrafish embryo toxicity test is performed as described in the SOP_ZFET_OECD_V02.8.
The materials and equipment described in the SOP have to be used. The test substance and controls are described in chapters 8 – 9.
Any deviation from the trial plan or the SOP must be reported.
For testing the transferability of the ZFET from the lead laboratory to the other participating laboratories, 3,4-dichloroaniline (3,4-DCA) is used as test chemical and tested in six concentrations as described in chapter 8. 3,4-DCA induces acute toxicity to zebrafish embryos and will be used as positive control during the other phases of the overall study.

Note: In this phase of the study, it will not be necessary to run a positive and a solvent control. However, a negative control is mandatory.

For all experiments, the plate layout shown in Annex 1 has to be used.

All experiments have to be recorded using the reporting template (*RT_ZFET_OECD_1a_V01.3_laboratory code_run*), which will be distributed by François Busquet to the participating laboratories.

7.2. Pre-saturation of glass vessels used for selection of fertilised eggs and 24-well plates

The 24-well plates and glass vessels must be pre-saturated with the respective concentrations of test substances and controls **24 hrs** before the day of the test. They are filled with the required quantity of freshly prepared test concentrations (freshly = prepared on the same day) and respective controls, e.g. glass vessels, at least 50 ml and 24-well plates, at least 2 ml/well (see also SOP_ZFET_OECD_V02.8; see *Note* in 6.3.2).

7.3. Daily semi-static renewal of test solutions/controls

Note: Analysis of the test concentrations at P&G showed a loss of more than 30% over the course of the test. The VMG therefore agreed that daily renewal of the test solutions/controls is mandatory.

Daily semi-static renewal of test solutions/controls should be performed according to SOP_ZFET_OECD_V02.8 section 6.5.

7.4. Measurements of test conditions

Measurements of test conditions should be performed according to SOP_ZFET_OECD_V02.8 section 6.6.

8. Test chemical

8.1. Information on 3,4-dichloroaniline

Name	3,4-dichloraniline
CAS	95-76-1
Supplier	Sigma-Aldrich (Fluka Pestanal® analytical standard)
Purchase number	35827
Lot number	6080X
Colour	Dark brown
Form	Solid
Purity (%)	99.9
Storage	room temperature
Molecular weight (g/mol)	162.02

The 3,4-dichloroaniline material safety data sheet is attached in Annex 3.

8.2. Preparation of 3,4-dichloroaniline stock solution (100mg/l)

a) Dissolve 50 mg 3,4-DCA in 500 ml dilution water

b) Stir in a closed, light-proof vessel for 24 h at room temperature

c) Adjust pH to the pH of the dilution water (within the range of ± 0.5)

d) Stock solution can be kept dark in refrigerator (1-8°C) for up to 6 months

e) Before use of the stock solution, stir at room temperature for at least 30 min to ensure a uniform concentration of the substance.

8.3. Test concentrations

a) The following concentrations of 3,4-DCA will be tested in Phase 1a:
0.5, 1.0, 2.0, 3.7, 4.0, 8.0 mg/l.

b) Test concentrations are freshly prepared (= on the same day) with dilution water (see 5.3 of SOP_ZFET_OECD_V02.8). The temperature of the dilution water should be 26±1˚C.

9. Controls

- Negative control
dilution water as described in SOP_ZFET_OECD_V02.8

- Positive control
not applicable in Phase 1a

- Solvent control
not applicable in Phase 1a

10. Shipping of stock solutions for analysis to P&G

Stock solutions of each lab will be analysed by P&G. In addition, P&G will analyse their own test concentrations. Details are given in Annex 3. The laboratories will be contacted by Scott Belanger regarding the necessary documents.

11. Reporting of results

The results (also of failed experiments) should be reported using the reporting template. The results are made available according to the deadlines given in chapter 5. A brief report summarising observations, deviations from SOP, comments etc should be added to the "remarks" sheet in the reporting template. The reporting templates are returned to François Busquet (e-mail: francois.busquet@jrc.ec.europa.eu).

12. Statistical analysis

An outline of the statistical data analysis is given in Annex 2.

13. Archiving

Reporting templates either filled in electronically, printed and signed, or handwritten, that are produced during the study are defined as raw data and should be archived by the participating laboratories.

14. Quality assurance statement

The participating laboratories should document their quality assurance system.

15. References

Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. & Seitz, N. (2005) Towards an alternative for the acute fish LC50 test in chemical assessment: The fish embryo toxicity test goes multi-species – an update. ALTEX 22: 87-102.

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.

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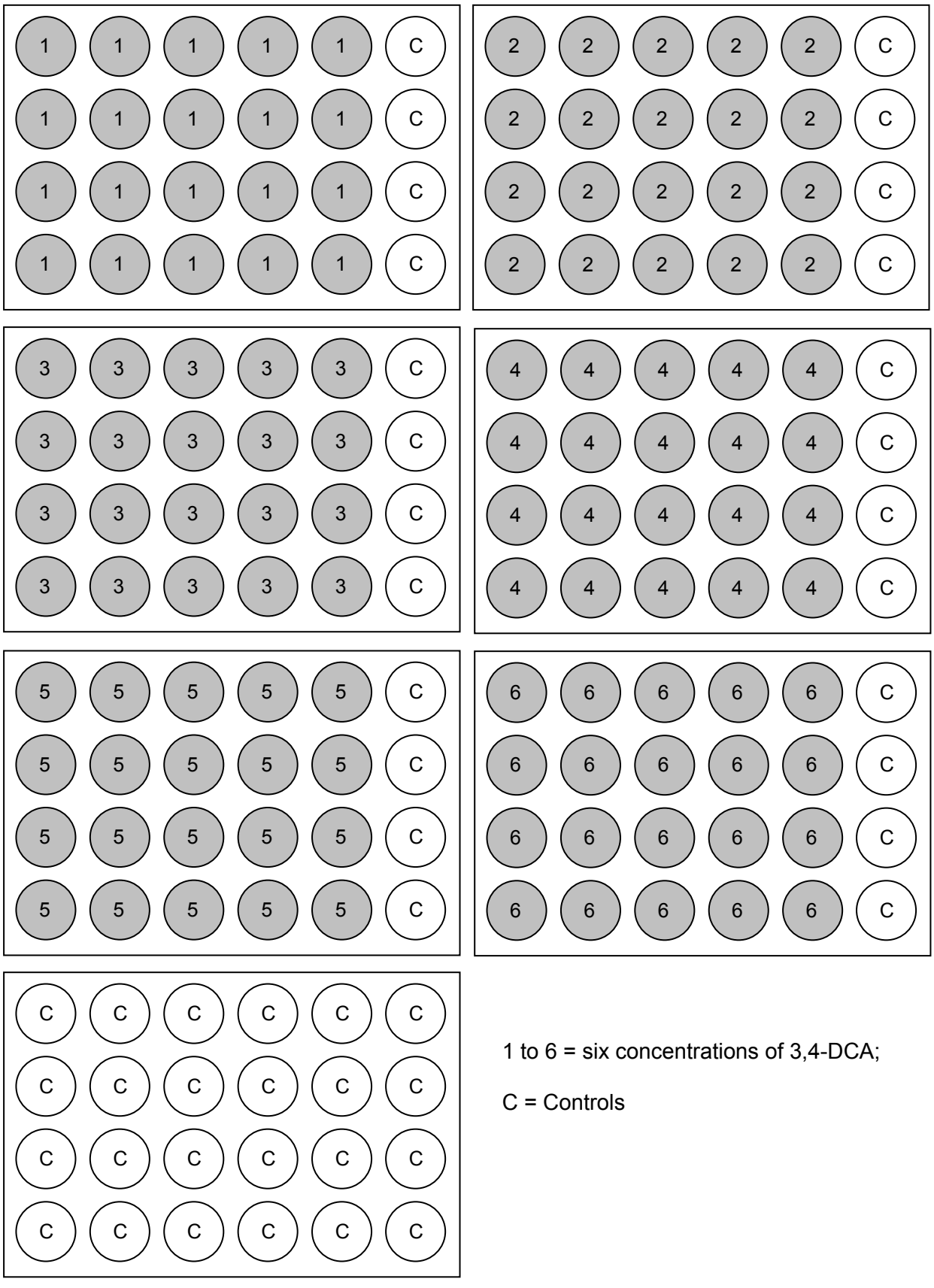
Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T. (2009) Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comparative Biochemistry and physiology, Part C, doi: 10.1016/j.cbpc.200811.006.

Nagel, R. (2002) DarT: The embryo test with the zebrafish Danio rerio – a general model in ecotoxicology and toxicology. ALTEX 19: 38-48.

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].

ANNEX 1: Layout of 24-well plates for Phase 1a



1 to 6 = six concentrations of 3,4-DCA;

C = Controls

ANNEX 2: Statistical analysis

Responsible – Andre Kleensang

As a basis, the following data analyses steps will be performed. Any deviations should be justified and explained in the report of the statistical data analysis. The analyses are not necessarily limited to the given steps.

1. Quality checks

1.1. Is the information complete?
1.2. Are acceptance criteria met?
1.3. Are reported results consistent? (i.e. Is an embryo reported as dead at 24 h still reported as dead at 96 h?)

2. Descriptive statistics

2.1. Summarise quality checks
2.2. Summarise results of chemical and control in tables and figures
2.3. Count failed (e.g. acceptance criteria not met (see 7.1 in SOP) or following the judgement of the operator) and summarise in tables
2.4. Summarise remarks

3. Inferential statistics

3.1. Choose appropriate model for estimating the LC50 including (robust) confidence intervals by following recommendations of the OECD Guidance Document No. 54 on current approaches in the statistical analysis of ecotoxicity data will be considered. As target one model should be chosen which showed an acceptable fit and robustness for all results.
3.2. Quality criteria for fitting a model:
- Do the assumptions of the model reflect the biological context?
- Inspection of residuals
- Transformations of the variables are indicated (e.g. log dose)?
- Convergence of maximization process

3.3. Estimate LC50 and confidence intervals per experiment
- Summarise model fits, quality criteria and confidence intervals
- Summarise dose-response curves in figures

3.4. Test of effect on internal controls caused by the increasing test concentrations using Cochran-Armitage trend test in a stratified manner (strata: laboratory).
3.5. Fisher test internal control vs. external control plate in a stratified manner (strata: laboratory).

4. Intralaboratory variability

4.1. Calculate coefficient of variation (CV) based upon LC50 estimates per lab. Will be performed on a log scale if necessary.
4.2. Fitting of a global random effects model on all LC50 estimates, on log scale (if necessary), which simultaneously estimates the components of variability due to within-lab replication, and between lab.

5. Interlaboratory variability

5.1. Calculate CV based upon the LC50 means estimate per lab. Will be performed on a log scale if necessary.

5.2. ANOVA and Post-hoc with laboratory (independent variable) vs. LC50 (depended variable).

5.3. See 4.2

6. Estimate possible concentration and acceptance criteria for test item as positive control for the next phases of the study

6.1. Plot figures with calculated LC's at different tested concentrations (as historical range)

6.2. Fit model with all valid data; calculate which dose shows lower border of 95% confidence interval at different LC's (e.g. 50, 75).

6.3. Remark: The approach used to estimate the possible concentration and acceptance criteria for test item as positive control for the next phases of the study will highly depend on the results of the study and can not be decided finally beforehand.

7. Report of statistical data analysis

The outcome will be summarised in a report to the validation management group.

8. Quality assurance of data analysis and reporting

An independent statistician (e.g. of IHCP) will review the data analysis and the report.

ANNEX 3:
Sampling, Shipment, and Receipt of Test Stock Solutions

The Analytical Laboratories of Procter & Gamble will be responsible for the determination of concentrations of test substances in stock solutions as part of the phase 1a.

Note:

Procter & Gamble will distribute instructions for shipment of sampled solutions to each participating laboratory identified in Section 4 of the Trial Plan for Phase 1a – Transferability. It is essential that these instructions are carefully followed in order to expedite export of the sample from the participating laboratory through import via United States Customs and subsequent delivery to Procter & Gambles laboratory. ***Failure to follow instructions exactly will likely result in the sample being held at EC and/or US Customs which could compromise sample integrity****.*

Two samples per stock solution per laboratory are requested. Sample 1 will be the primary sample for analysis and Sample 2 will serve as a back-up in reserve in the case of spillage or other laboratory issue.

1. Labeling Sample Containers:

- Samples should be clearly and legibly labeled with the following information at a minimum:
  - Researcher name
  - Laboratory name
  - Material name and CASNO
  - Nominal concentration of sample
  - Date sample was taken
  - Type of sample (i.e., stock solution)
  - Sample code (consisting of two letter location indicator, date on DDMMYY format followed by -1 or -2 as further described below)
    - An example code from Procter & Gamble's Aquatic Toxicology Laboratory may look like "PG030509-1" for a sample taken by Procter & Gamble on 3 May 2009, sample 1).

2. Sample Containers:
- Use amber borosilicate glass, VWR catalogue 80076-572 or similar (e.g., Wheaton #W224604), with screw caps (solid-top lined with PTFE faced 14B white styrene-butadiene rubber).
- Minimum volume 10 mL, maximum volume 20 mL
- Pre-rinse any sample container with an initial sample
- Fill container completely and cap
- Wrap cap with Parafilm or equivalent
- Wrap entire sample in aluminum foil

3. Packing of samples
   - Follow all necessary packing instructions as indicated on Material Safety Data Sheet (MSDS)
   - Provide MSDS with the shipped samples
   - Complete a Chain of Custody for each shipment. This paperwork will travel with the sample through all phases of analysis and reporting.

Shipping Samples

- Shipping documents with import/export instructions will be sent to each participating laboratory, the analytical contact at Procter & Gamble (Dr. Ken Wehmeyer) and the ecotoxicology laboratory at Procter & Gamble (Dr. Scott Belanger). It is imperative to send samples with this paperwork. Use of carriers certified for handling hazardous substances is required and will be explained in the paperwork (note that most international carriers are certified for this purpose).
- Shipments of samples are to be sent to:
  Dr. Ken Wehmeyer
  Procter & Gamble, 8700 Mason-Montgomery Road
  Mason, Oh 45040 USA
  Tel : 513-622-2149
  FAX : 513-622-0523
  Email: wehmeyer.kr@pg.com
- Each laboratory should notify Scott Belanger at Belanger.se@pg.com that paperwork has been received.
- Upon shipment, notify Scott Belanger again that the shipment is en route.
  Note: Include the Shipper used (e.g., FedEx, DHL, etc.), the date the sample left the laboratory, and a copy of the shipment invoice and tracking number.
- Procter & Gamble will notify laboratories when samples are received.

Two replicate samples in separate containers are requested for each stock solution sampled. Samples should be sent in the same parcel.

Analysis will be conducted by Procter & Gamble in the laboratory of Dr. Ken Wehmeyer by a scientifically valid and documented method, but not under full GLP-compliance. Standard curves, repeatability, instrument conditions, other relevant specifications, notebook and technical oversight will be recorded. A single report will be prepared for each group of assays conducted at a timing that will be at the discretion of Dr. Wehmeyer's laboratory. Samples will be grouped and run as one as much as possible.

Material Safety Data Sheet

Version 3.0
Revision Date 08/23/2008
Print Date 02/18/2009

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : 3,4-Dichloroaniline

Product Number : 35827
Brand : Fluka

Company : Sigma-Aldrich
3050 Spruce Street
SAINT LOUIS MO 63103
USA
Telephone : +1 800-325-5832
Fax : +1 800-325-5052
Emergency Phone # : (314) 776-6555

2. COMPOSITION/INFORMATION ON INGREDIENTS

Formula : C6H5Cl2N
Molecular Weight : 162.02 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
3,4-Dichloroaniline			
95-76-1	202-448-4	612-202-00-1	-

3. HAZARDS IDENTIFICATION

Emergency Overview

OSHA Hazards
Target Organ Effect, Highly toxic by inhalation, Harmful by ingestion., Toxic by skin absorption, Skin sensitizer, Corrosive

Target Organs

Blood

HMIS Classification

Health Hazard: 3
Flammability: 0
Physical hazards: 0

NFPA Rating

Health Hazard: 3
Fire: 0
Reactivity Hazard: 0

Potential Health Effects

Inhalation May be fatal if inhaled. Material is extremely destructive to the tissue of the mucous membranes and upper respiratory tract.

Skin	Toxic if absorbed through skin. Causes skin burns.
Eyes	Causes eye burns.
Ingestion	Harmful if swallowed. Causes burns.

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing give artificial respiration Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.

In case of eye contact
Continue rinsing eyes during transport to hospital.Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.

5. FIRE-FIGHTING MEASURES

Flammable properties

Flash point	135.00 °C (275.00 °F) - closed cup
Ignition temperature	265 °C (509 °F)

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Wear respiratory protection. Avoid dust formation. Avoid breathing dust. Ensure adequate ventilation. Evacuate personnel to safe areas.

Environmental precautions
Prevent further leakage or spillage if safe to do so. Do not let product enter drains. Discharge into the environment must be avoided.

Methods for cleaning up
Pick up and arrange disposal without creating dust. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Storage
Keep container tightly closed in a dry and well-ventilated place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Components with workplace control parameters

Components	CAS-No.	Value	Control parameters	Update	Basis
3,4-Dichloroaniline	95-76-1	TWA	5 ppm 19 mg/m3	1993-06-30	US. Department of Labor - Occupational Safety and Health Administration (OSHA) Permissible Exposure Limits (PEL) 29 CFR 1910.1000 Air Contaminants.
Remarks	Skin contact does contribute to exposure.				
		TWA	2 ppm 8 mg/m3	1989-03-01	US. Department of Labor - Occupational Safety and Health Administration (OSHA) 29 CFR 1910.1000 Z-1-A
	Skin contact does contribute to exposure.				

Personal protective equipment

Respiratory protection
Where risk assessment shows air-purifying respirators are appropriate use a full-face particle respirator type N100 (US) or type P3 (EN 143) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator. Where risk assessment shows air-purifying respirators are appropriate use a dust mask type N95 (US) or type P1 (EN 143) respirator. Where risk assessment shows air-purifying respirators are appropriate use a full-face particle respirator type N99 (US) or type P2 (EN 143) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves.

Eye protection
Safety glasses

Skin and body protection
Choose body protection according to the amount and concentration of the dangerous substance at the work place.

Hygiene measures
Avoid contact with skin, eyes and clothing. Wash hands before breaks and immediately after handling the product.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid
Colour	dark brown

Safety data

pH	no data available
Melting point	70 °C (158 °F)
Boiling point	272 °C (522 °F) at 1,013 hPa (760 mmHg)

Flash point	135.00 °C (275.00 °F) - closed cup
Ignition temperature	265 °C (509 °F)
Lower explosion limit	2.8 %(V)
Upper explosion limit	7.2 %(V)
Water solubility	no data available
Relative vapour density	6.49

10. STABILITY AND REACTIVITY

Storage stability
Stable under recommended storage conditions.

Materials to avoid
Acid anhydrides, Oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Hydrogen chloride gas

11. TOXICOLOGICAL INFORMATION

Acute toxicity

LD50 Oral - rat - 545 mg/kg

Irritation and corrosion

Skin - rabbit - Severe skin irritation

Eyes - rabbit - Severe eye irritation

Sensitisation

May cause allergic skin reaction.

Causes sensitization.

Chronic exposure

IARC:	No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.
ACGIH:	No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.
NTP:	No component of this product present at levels greater than or equal to 0.1% is identified as a known or anticipated carcinogen by NTP.
OSHA:	No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by OSHA.

Genotoxicity in vitro - Human - lymphocyte
Sister chromatid exchange

Potential Health Effects

Inhalation	May be fatal if inhaled. Material is extremely destructive to the tissue of the mucous membranes and upper respiratory tract.
Skin	Toxic if absorbed through skin. Causes skin burns.
Eyes	Causes eye burns.
Ingestion	Harmful if swallowed. Causes burns.

Target Organs Blood,

Additional Information
RTECS: BX2625000

12. ECOLOGICAL INFORMATION

Elimination information (persistence and degradability)

Biodegradability	Result: - Not readily biodegradable.
Bioaccumulation	Poecilia retiaculata (guppy) - 48 h Bioconcentration factor (BCF): 96

Ecotoxicity effects

Toxicity to fish	LC50 - Pimephales promelas (fathead minnow) - 7 - 10 mg/l - 96 h
Toxicity to daphnia and other aquatic invertebrates.	EC50 - Daphnia magna (Water flea) - 0.05 - 2.20 mg/l - 48 h
Toxicity to algae	EC50 - Pseudokirchneriella subcapitata (green algae) - 4.9 mg/l - 72 h
	Growth inhibition LOEC - Algae - 1 - 10 mg/l - 28 d

Further information on ecology

An environmental hazard cannot be excluded in the event of unprofessional handling or disposal.

Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.

13. DISPOSAL CONSIDERATIONS

Product
Observe all federal, state, and local environmental regulations. Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
UN-Number: 3442 Class: 6.1 Packing group: II
Proper shipping name: Dichloroanilines, solid
Marine pollutant: Marine pollutant
Poison Inhalation Hazard: No

IMDG
UN-Number: 3442 Class: 6.1 Packing group: II EMS-No: F-A, S-A
Proper shipping name: DICHLOROANILINES, SOLID
Marine pollutant: Marine pollutant

IATA
UN-Number: 3442 Class: 6.1 Packing group: II
Proper shipping name: Dichloroanilines, solid

15. REGULATORY INFORMATION

OSHA Hazards
Target Organ Effect, Highly toxic by inhalation, Harmful by ingestion., Toxic by skin absorption, Skin sensitizer, Corrosive

DSL Status
All components of this product are on the Canadian DSL list.

SARA 302 Components
SARA 302: No chemicals in this material are subject to the reporting requirements of SARA Title III, Section 302.

SARA 313 Components
SARA 313: This material does not contain any chemical components with known CAS numbers that exceed the threshold (De Minimis) reporting levels established by SARA Title III, Section 313.

SARA 311/312 Hazards
Acute Health Hazard, Chronic Health Hazard

Massachusetts Right To Know Components

	CAS-No.	Revision Date
3,4-Dichloroaniline	95-76-1	1991-07-01

Pennsylvania Right To Know Components

	CAS-No.	Revision Date
3,4-Dichloroaniline	95-76-1	1991-07-01

New Jersey Right To Know Components

	CAS-No.	Revision Date
3,4-Dichloroaniline	95-76-1	1991-07-01

California Prop. 65 Components
This product does not contain any chemicals known to State of California to cause cancer, birth, or any other reproductive defects.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

TEST SAMPLE COLLECTION AND CHAIN OF CUSTODY

Page_____ of _____

Study Name/Number:_____ **Lab Destination:**_____

Sample Team Members:_____ **Carrier/Waybill #:**_____

Sample Number	Sample Location or Description	Initials/Date/Time	Container Type

Total Number of Samples_____

Special Instructions_____

Possible Hazards_____

Sample Transfer and Custody Record (Signatures, Company, Date, and Time)

1. Relinquished By:_____
   Received By:_____

2. Relinquished By:_____
   Received By:_____

3. Relinquished By:_____
   Received By:_____

4. Relinquished By:_____
   Received By:_____

5. Relinquished By:_____
   Received By:_____

PHASE 1a - PARTICIPATING LABORATORIES	
All samples shall be sent to: Dr. Ken Wehmeyer Procter & Gamble, 8700 Mason-Montgomery Road Mason, Oh 45040 USA **Tel** : 513-622-2149 **FAX** : 513-622-0523 **Email:** wehmeyer.kr@pg.com **Alternate P&G contact:** Mike Karb **Tel:** 513-622-2260 Email: karb.mj@pg.com (all other details remain the same)	
Notification of sample shipments should also be made by email to: Scott E. Belanger, PhD Email: Belanger.se@pg.com	
1st contact	**2nd contact**
Prof. Dr. Thomas Braunbeck Aquatic Ecology and Toxicology Section Dept. of Zoology, University of Heidelberg Im Neuenheimer Feld 230 D-69120 Heidelberg GERMANY Tel: +49 6221 545668 Fax: +49-6221-546162 braunbeck@zoo.uni-heidelberg.de	Dipl.-Biol. Ruben Strecker Aquatic Ecology and Toxicology Section Dept. of Zoology, University of Heidelberg Im Neuenheimer Feld 230 D-69120 Heidelberg GERMANY Tel: +49 6221 546255 Fax: +49-6221-546162 Ruben.Strecker@zoo.uni-heidelberg.de
Przemysław Fochtman, PhD Deputy Head of the Branch for Scientific Affairs Institute of Industrial Organic Chemistry Branch Pszczyna Ul. Doswiadczalna 27 43-200 Pszczyna POLAND Tel: +48 32 210 30 81 Fax: 48 32 210 35 37 E-mail: fochtman@ipo-pszczyna.pl	Helena Rzodeczko, MSc Institute of Industrial Organic Chemistry Branch Pszczyna Ul. Doswiadczalna 27 43-200 Pszczyna POLAND Tel: +48 32 210 30 81 Fax: 48 32 210 35 37 E-mail: ew@ipo-pszczyna.pl
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Leo van der Ven, PhD Laboratory for Health Protection Research National Institute of Public Health and the Environment RIVM, GBO 12 PO Box 1 NL-3720 BA Bilthoven THE NETHERLANDS Tel: +31 30 274 2681 Fax: +31 30 274 4446 Leo.van.der.Ven@rivm.nl	Evert-Jan van den Brandhof Laboratory for Ecological Risk Assessment National Institute of Public Health and the Environment RIVM, LER 9 PO Box 1 NL-3720 BA Bilthoven THE NETHERLANDS Tel: +31 30 274 3544 Fax: +31 30 274 4413 Evert-Jan.van.den.Brandhof@rivm.nl
Hilda Witters, PhD VITO Flemish Institute for Technological Research Unit Environmental Health and Risk Research team Toxicology Administration: Boeretang 200 B-2400 Mol BELGIUM Tel: +32 14 335213 Fax: 32-14-582657 hilda.witters@vito.be **Laboratory = address delivery of chemicals** VITO Research team Toxicology Retieseweg (across n° 138) B-2440 Geel Belgium Tel: +32 14 335213 Fax: 32-14-582657 hilda.witters@vito.be	Ingrid Selderslaghs VITO Flemish Institute for Technological Research Unit Environmental Health and Risk Research team Toxicology Administration: Boeretang 200 B-2400 Mol BELGIUM Tel: +32 14 335261 Fax: 32-14-582657 Ingrid.selderslaghs@vito.be **Laboratory = address delivery chemicals** VITO Research team Toxicology Retieseweg (across n° 138) B-2440 Geel Belgium Tel: +32 14 335261 Fax: 32-14-582657 Ingrid.selderslaghs@vito.be

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

Title	**Zebrafish Embryo Toxicity Test: Evaluation of transferability, intra- and interlaboratory reproducibility – Phase 1b: Testing of six chemicals**		
Sponsor:	**-**		
Identification:	*TP_ZFET_OECD_1b*		
Start Date:	**13 November 2009**	**End Date:**	**28 February 2010**

Trial Coordinator	Name	Marlies Halder
	Date	November 2009
	Signature	
Reviewer 1	Name	Participating Laboratories
	Date	
	Signature	
Reviewer 2	Name	VMG
	Date	
	Signature	

PAGES OF CHANGES

Date of change	Version number	Changed pages/sections	Summary of the change(s)	Changed by/sign

Table of Contents

Zebrafish Embryo Toxicity Test
Evaluation of transferability, intra- and interlaboratory reproducibility

Phase 1b: Testing of six chemicals

1. Introduction

The acute fish toxicity test is a mandatory component in the environmental safety assessment of industrial chemicals, agrochemicals, pharmaceuticals, feed stuff etc. In the European Union, Council Directive 86/609/EEC on the protection of laboratory animals (EC, 1986) and, in particular, the legislation on chemicals (REACH; EC 2007) demand that tests on vertebrate animals are reduced, refined or replaced whenever possible.

One of the most promising alternative approaches to the LC50 96h fish toxicity test (OECD 203 [OECD, 1992]; C.1 [EC, 2008]) is based on the use of fish embryos.

In Germany, the Fish Egg Toxicity test (DIN 2001) was validated and replaced the 48 h acute fish test for routine whole effluent testing in 2005. Recently, a modified international version of the fish egg toxicity test was published (ISO 2007).

Extensive efforts have been undertaken to adapt the method to also meet chemical testing requirements (Nagel 2002, Braunbeck *et al.,* 2005, Lammer *et al.,* 2009). In fall 2005, the German Federal Environment Agency submitted the draft guideline “Fish embryo toxicity (FET) test” to the OECD Test Guideline program 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 teleconference 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 demonstrates that the zebrafish fish embryo test correlates well with acute fish toxicity tests (Lammer *et al.* 2009).

The ad hoc Expert Group on the Fish Embryo Toxicity Test noted that most data are available for the zebrafish embryo toxicity test, however, data providing sufficient evidence for the reproducibility of the method are lacking.

2. Purpose of the study

The zebrafish embryo toxicity test (ZFET) is designed to determine the lethal effects of chemicals on embryonic stages of fish and constitutes an alternative test method to the acute toxicity tests with juvenile and adult fish, i.e. the OECD Test Guideline 203 (OECD 1992).

Following the advice of the OECD ad hoc Expert Group on Fish Embryo Tests, OECD decided to perform a ring trial in a restricted number of laboratories. The purpose is to evaluate:

- the transferability,
- the intralaboratory reproducibility, and
- the interlaboratory reproducibility of the ZFET.

The study is steered by a validation management group.

The study is divided into two phases, where Phase 1 constitutes the transferability of the ZFET from the Lead laboratory to the other laboratories (Phase 1a – Transferability/Training) and consequent the testing of six chemicals (Phase 1b). Based on the outcome of Phases 1a and 1b, the standard operation procedure (SOP) might undergo revisions. In Phase 2, a larger set of substances will be tested.

3. Validation management group

The validation management group (VMG) will steer the study and is responsible for the overall study design. Specific roles and responsibilities are listed below:

Name	**Affiliation/contact**	**Role**
Marlies Halder François Busquet	JRC/IHCP/IVM-ECVAM marlies.halder@jrc.ec.europa.eu francois.busquet@jrc.ec.europa.eu	Coordination/reporting
André Kleensang	JRC/IHCP/IVM-ECVAM andre.kleensang@jrc.ec.europa.eu	Data analysis
Patric Amcoff	OECD patric.amcoff@oecd.org	OECD TG Program
Thomas Braunbeck	University of Heidelberg braunbeck@zoo.uni-heidelberg.de	Lead laboratory, SOP; UBA representative
Scott Belanger	Procter & Gamble belanger.se@pg.com	Chemical analysis, participating laboratory
Adam Lillicrap	NIVA Adam.Lillicrap@niva.no	Independent adviser

4. Participating laboratories

Name	responsible/contact	Role
University of Heidelberg	Thomas Braunbeck braunbeck@zoo.uni-heidelberg.de	Lead laboratory
Procter & Gamble	Scott Belanger belanger.se@pg.com	Participating laboratory
IVM	Juliette Legler juliette.legler@ivm.vu.nl	Participating laboratory
UFZ	Stefan Scholz Stefan.Scholz@ufz.de	Participating laboratory
RIVM	Leo van der Ven Leo.van.der.Ven@rivm.nl	Participating laboratory
VITO	Hilda Witters hilda.witters@vito.be	Participating laboratory

Full contact details and alternate person to be contacted are given in Annex 4.

5. Standard operation procedure

The use of the SOP_ZFET_OECD_V02.9 is mandatory. Any deviation from the SOP must be reported in the reporting template.

6. Time schedule and design of the study

The study design covers testing of 6 chemicals by the participating laboratories.

Table 1: Study design

Week	Step	Action	Responsible
	0	Distribution of draft documents for Phase 1b, i.e. trial plan, SOP, reporting templates via e-mail – labs should carefully read the documents and contact ECVAM if explanations are required	ECVAM
1	1	Distribution of final documents for Phase 1b, i.e. trial plan, SOP, reporting templates via e-mail – labs confirm receipt	ECVAM
1	2	Distribution of the 6 test chemicals and MSDS as indicated in Table 2 – labs confirm receipt	ECVAM
	3	a) A new stock solution of 3,4 DCA must be prepared (see section 9.2) b) Preparation of stock solutions of test chemicals as described in section 8 c) Sampling and storage of stock solutions as described in section 7.6 d) Testing of test chemicals (5 concentrations) in 3 independent runs with the appropriate controls (see sections 8-9). e) Submission of data to ECVAM	Participating labs
	4	Analytical measurements of stock solutions and P&G test concentrations	P&G
	5	Analysis of data	ECVAM
	6	Discussion of results & decision on progression to Phase 2	VMG

7. Study performance

7.1. General considerations

The zebrafish embryo toxicity test is performed as described in the SOP_ZFET_OECD_V02.9.
The materials and equipment described in the SOP have to be used. The test substances and controls are described in section 8 – 9.
Any deviation from the trial plan or the SOP must be reported.

For all experiments, the plate layout shown in Annex 1 has to be used.

All experiments have to be recorded using the reporting template (*RT_ZFET_OECD_1b_V01.1_Laboratory code_Chemical_Run*), which will be distributed by ECVAM to the participating laboratories.

7.2. Chemicals tested per laboratory

Note: Since not all of the 6 laboratories have the capacity to test all of the 6 chemicals in 3 independent runs, the VMG decided to distribute the chemicals amongst the 6 laboratories as given in Table 2. This distribution ensures that each chemical is at least tested in 4 laboratories.

Table 2: Chemicals to be tested by participating laboratories

		VITO	RIVM	UFZ	IVM	Heidelberg	P&G
Non Toxic	**Ethanol**	X		X	X	X	X
	Sodium chloride		X	X		X	X
Moderately Toxic	**2,3,6 Trimethylphenol**	X		X	X	X	X
	6-Methyl-5-heptene-2-one		X	X		X	X
Toxic	**Dibutyl maleate**	X		X	X	X	X
	Triclosan		X	X		X	X

7.3. Pre-saturation of glass vessels used for selection of fertilised eggs and 24-well plates

The 24-well plates and glass vessels must be pre-saturated with the respective concentrations of test substances and controls at least **24 hrs** before the day of the test.

They are filled with the required quantity of freshly prepared test concentrations (freshly = prepared on the same day) and respective controls, e.g. glass vessels, at least 50 mL and 24-well plates, at least 2 mL/well (see also *Note* in 6.3.2 of SOP_ZFET_OECD_V02.9).

7.4. Daily semi-static renewal of test solutions/controls

Daily semi-static renewal of test solutions/controls should be performed according to SOP_ZFET_OECD_V02.9 section 6.5.

7.5. Measurements of test conditions

Measurements of test conditions should be performed according to SOP_ZFET_OECD_V02.9 section 6.6.

7.6. Analytical measurements of stock solutions

As indicated in Table 3:

- For 4 chemicals (Dibutyl maleate, 2,3,6 Trimethylphenol, Triclosan and 6-Methyl-5-heptene-2-one), measurements will be carried out by Procter and Gamble. Stock solutions should be sampled and stored for analysis as described in section 8 and Annex 3.
- Measurements of stock solutions of **sodium chloride** will be performed by the laboratories as described in section 8.4.2).
- No measurement is required for ethanol.

Table 3: Analysis of stock solutions

	Laboratories	**P&G**
Ethanol	**-**	**-**
Sodium chloride	**X**	**-**
2,3,6 Trimethylphenol		**X**
6-methyl-5-heptene-2-one		**X**
Dibutyl maleate		**X**
Triclosan		**X**

8. Test chemicals

Note: ECVAM has purchased the 6 test chemicals and will distribute them to the laboratories with the Material Safety Data Sheets (MSDS).
Laboratories will be informed by ECVAM on the date of sending, the tracking number, and the number of samples.
On receipt, laboratories must control the status of the samples and report back to ECVAM (e-mail to François Busquet).

8.1. Dibutyl maleate

8.1.1. Information on dibutyl maleate

Name	Dibutyl maleate
CAS	105-76-0
Supplier	Aldrich
Purchase number	D47102
Lot number	07715ch
Colour	colourless
Form	liquid
Purity (%)	97.8 (gas liquid)
Storage	cool place
Molecular weight (g/mol)	228.28

8.1.2. Preparation of Dibutyl maleate stock solution (50mg/L)

Note: The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

- Dissolve 50 mg Dibutyl maleate in 1L of dilution water. The substance is a liquid, hence a calculation via the density (0.994 g/cm³) is needed. 50 mg correspond to 50 µL of the substance.
- Stir in a closed, light proof vessel for 30 minutes at room temperature to ensure that the Dibutyl maleate is completely dissolved.
- Adjust pH to the pH of the dilution water (± 0.5).
- The stock solution can be kept refrigerated in the dark (1-8°C) during a **single** run.
- Before use of the stock solution, stir at room temperature for 30 min to ensure uniform concentration of the substance.
- Of each stock solution prepared two samples should be preserved 1:1 with methanol (for example, 5 mL stock, 5 mL methanol) and stored at a cool place (see Annex 3 for details of sampling).

8.1.3. Dibutyl maleate test concentrations

- The following concentrations of Dibutyl maleate will be tested in Phase 1b:
  0.25, 0.5, 1.0, 2.0, and 4.0 mg/L

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.9). The temperature of the dilution water should be 26±1°C.

8.2. Ethanol

8.2.1. Information on ethanol

Name	Ethanol
CAS	64-17-5
Supplier	Sigma
Purchase number	34923
Lot number	sze91380
Colour	colourless
Form	liquid
Purity (%)	≥99.9 (GC)
Storage	cool place; hygroscopic
Molecular weight (g/mol)	46.07

8.2.2. Preparation of ethanol stock solution

- Use pure ethanol for preparing the respective concentrations.

8.2.3. Ethanol test concentrations

- The following concentrations of ethanol will be tested in Phase 1b:
  5.3, 8, 12, 18, 27 g/L

- Prepare test concentration as given in Table 4. The substance is a liquid, hence calculation is based on the density (0.79 g/cm^3).

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.9). The temperature of the dilution water should be 26±1°C.

Table 4:Preparation of ethanol test concentrations

Test concentrations (g/L)	Volume of ethanol to be added (mL)	Volume of dilution water to be added (mL)
5.3	0.336	49.664
8	0.507	49.493
12	0.761	49.239
18	1.141	48.859
27	1.711	48.289

8.3. 6-Methyl-5-heptene-2-one

8.3.1. Information on 6-Methyl-5-heptene-2-one

Name	6-Methyl-5-heptene-2-one
CAS	110-93-0
Supplier	Fluka
Purchase number	67320
Lot number	S52972-229
Colour	colourless
Form	liquid
Purity (%)	96 (GC)
Storage	cool place
Molecular weight (g/mol)	132.8

8.3.2. Preparation of 6-Methyl-5-heptene-2-one stock solution

Note: The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

- Dissolve 1000 mg 6-Methyl-5-heptene-2-one in 1L of dilution water. The substance is a liquid, hence a calculation via the density (0.852 g/cm³) is needed. 1000 mg correspond to 1174 µL of the substance.
- Stir 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.
- Adjust pH to the pH of the dilution water (± 0.5).
- The stock solution can be kept refrigerated in the dark (1-8°C) during a single run.
- Before use of the stock solution, stir at room temperature for 30 min to ensure uniform concentration of the substance.
- Of each stock solution prepared two samples should be stored at -20°C and shipped on dry ice (see Annex 3 for details of sampling).

8.3.3. 6-Methyl-5-heptene-2-one test concentrations

- The following concentrations of 6-Methyl-5-heptene-2-one will be tested in Phase 1b: **25, 42.5, 72.25, 122.825, 208.03 mg/L**

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.9). The temperature of the dilution water should be 26±1°C.

8.4. Sodium chloride

8.4.1. Information on sodium chloride

Name	Sodium chloride
CAS	7647-14-5
Supplier	Sigma
Purchase number	S7653
Lot number	106K0081
Colour	white
Form	powder
Purity (%)	100 (titration)
Storage	cool place
Molecular weight (g/mol)	58.44

8.4.2. Preparation of sodium chloride stock solution (50g/L)

- Dissolve 50g sodium chloride in 1L of dilution water.

- Stir for 30 minutes at room temperature to ensure the sodium chloride is completely dissolved.

- Adjust pH to the pH of the dilution water (± 0.5) if needed.

- The stock solution can be kept at room temperature in a closed container because it is not subject to any degradative loss.

- Before use of the stock solution, stir for 30 min to ensure uniform concentration of the substance.

Note: Concentration of stock solutions should be confirmed by measuring conductivity or salinity by each laboratory. Results should be reported in the cover sheet of the reporting template.

8.4.3. Sodium chloride test concentrations

- The following concentrations of sodium chloride will be tested in Phase 1b: **1.0, 2.0, 4.0, 8.0 and 16.0 g/L**

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.9). The temperature of the dilution water should be 26±1°C.

8.5. Triclosan (irgasan)

8.5.1. Information on Triclosan (irgasan; 5-chloro-2-2(2,4-dichlorophenoxy)phenol)

Name	Triclosan
CAS	3380-34-5
Supplier	Sigma
Purchase number	72779
Lot number	1412854
Colour	white
Form	powder with lumps
Purity (%)	99.7 (HPLC)
Storage	cool place
Molecular weight (g/mol)	289.54

8.5.2. Preparation of Triclosan stock solution (1.2g/L)

Note: Ethanol used to dissolve Triclosan will not be provided.

- Dissolve 120 mg Triclosan in 100 mL of ethanol (200 proof, ACS/USP grade).

- Stir in a closed, light proof vessel for 30 minutes at room temperature to ensure the Triclosan is completely dissolved.

- Adjustment of pH is not needed.

- The stock solution can be kept in the refrigerator (1-8°C) for several weeks.

- Before use of the stock solution, stir at room temperature for 30 min to ensure uniform concentration of the substance.

- Of each stock solution prepared two samples should be stored at 1-8°C (see Annex 3 for details of sampling).

8.5.3. Triclosan test concentrations

- The following concentrations of Triclosan will be tested in Phase 1b: **0.075, 0.15, 0.30, 0.60 and 1.20 mg/L**

- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.9). The temperature of the dilution water should be 26±1°C.

- The final concentration of ethanol should be 0.1 % in all the test concentrations and the dilution scheme given in Table 5 should be followed.

- Tests must be run with a solvent control (see section 9.3) and prepared according to Table 5.

Table 5: Triclosan test concentrations preparation

Test concentration (mg/L)	Volume of Triclosan stock solution to be added (mL)	Volume of ethanol to be added (mL)	Volume of dilution water to be added (mL)
0.075	0.063	0.937	999
0.15	0.125	0.875	999
0.3	0.250	0.750	999
0.6	0.500	0.500	999
1.2	1.000	0	999
Solvent control	0	1.000	999

8.6. 2,3,6 Trimethylphenol

8.6.1. Information on 2,3,6 Trimethylphenol

Name	2,3,6 Trimethylphenol
CAS	2416-94-6
Supplier	Fluka
Purchase number	92693
Lot number	1290095
Colour	slightly yellow
Form	solidified mass or chunks
Purity (%)	99.6 (GC)
Storage	cool place
Molecular weight (g/mol)	136.19

8.6.2. Preparation of 2,3,6 Trimethylphenol stock solution (250mg/L)

Note: The long term stability of this compound is not known under storage conditions; therefore, a new stock solution must be prepared for each run.

- Dissolve 250 mg 2,3,6 Trimethylphenol in 1L of dilution water.
- Stir in a closed, light proof vessel for at least 4 h at room temperature to ensure the Trimethylphenol is completely dissolved.
- Adjust pH to the pH of the dilution water (± 0.5).
- The long term stability of this compound is not known under storage conditions; therefore, stock solutions should be made for each run.
- The stock solution can be kept refrigerated in the dark (1-8°C) during a single run.
- Before use of the stock solution, stir at room temperature for 30 min to ensure uniform concentration of the substance.
- Of each stock solution prepared two samples should be stored at -20°C and shipped on dry ice (see Annex 3 for details of sampling).

8.6.3. 2,3,6 Trimethylphenol test concentrations

- The following concentrations of 2,3,6 Trimethylphenol will be tested in Phase 1b: **8, 12, 18, 27, 40.5 mg/L**
- Test concentrations are prepared fresh (on the same day) with dilution water (see section 5.3 of SOP_ZFET_OECD_V02.9). The temperature of the dilution water should be 26 ± 1°C.

9. Controls

9.1. Negative control

Dilution water (described in SOP_ZFET_OECD_V02.9) is used for internal and external negative controls (see Annex 1 for the layout of 24-well plates).

9.2. *Positive control*

3,4-dichloraniline is used as positive control (see Annex 1 for the layout of 24-well plates).

9.2.1. Information on 3,4-Dichloroaniline

Name	3,4-dichloraniline
CAS	95-76-1
Supplier	Sigma-Aldrich (Fluka Pestanal® analytical standard)
Purchase number	35827
Lot number	6080X
Colour	dark brown
Form	solid
Purity (%)	99.9
Storage	room temperature
Molecular weight (g/mol)	162.02

***Note**: The material safety data sheet for 3,4-Dichloroaniline is provided in TP_ZFET_OECD_1a_V01.7.*

9.2.2. Preparation of 3,4-Dichloroaniline stock solution (100mg/L)

- Dissolve 50 mg 3,4-Dichloroaniline in 500 mL dilution water.
- Stir in a closed, light-proof vessel for 24 h at room temperature.
- Adjust pH to the pH of the dilution water (within the range of ± 0.5).
- Stock solution can be kept dark in refrigerator (1-8°C) for up to 2 months.
- Before use of the stock solution, stir at room temperature for at least 30 min to ensure a uniform concentration of the substance.

9.2.3. Concentration of positive control

A concentration of ***4.0 mg/L*** of 3,4-Dichloroaniline will be used as a positive control.

The 3,4-Dichloroaniline solution is freshly prepared (= on the same day) with dilution water (see 5.3 of SOP_ZFET_OECD_V02.9). The temperature of the dilution water should be 26±1˚C.

9.3. Solvent control

The preparation of Triclosan (irgasan) requires the uses of a solubilising agent (ethanol).

A solvent control (see Annex 1 for the layout of 24-well plates) with ethanol is prepared as described in Table 5.

10. Shipping of stock solutions for analysis to P&G

Information on shipment will be provided as soon as available.

11. Reporting of results

The results (also of failed experiments) should be reported using the reporting template. The results are made available according to the deadlines given in section 5. A brief report summarising observations, deviations from SOP, comments etc should be added to the "remarks" sheet in the reporting template. The reporting templates are returned to François Busquet (e-mail: francois.busquet@jrc.ec.europa.eu).

12. Statistical analysis

An outline of the statistical data analysis is given in Annex 2.

13. Archiving

Reporting templates either filled in electronically, printed and signed, or handwritten, that are produced during the study are defined as raw data and should be archived by the participating laboratories.

14. Quality assurance statement

The participating laboratories should document their quality assurance system.

15. References

Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. & Seitz, N. (2005) Towards an alternative for the acute fish LC50 test in chemical assessment: The fish embryo toxicity test goes multi-species – an update. ALTEX 22: 87-102.

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.

European Commission (1986). Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. Official Journal of the European Communities L358, 1-29.

European Commission, 2007. Corrigendum to Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal of the European Union. L136, 3-282.

European Commission, 2008. Regulation (EC) No 440/2008. Laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). C.1. Acute Toxicity For Fish. Official Journal of European Union. L142, 446-455.

ISO (2007) Water quality – Determination of the acute toxicity of waste water to zebrafish eggs (Danio rerio). ISO 15088:2007.

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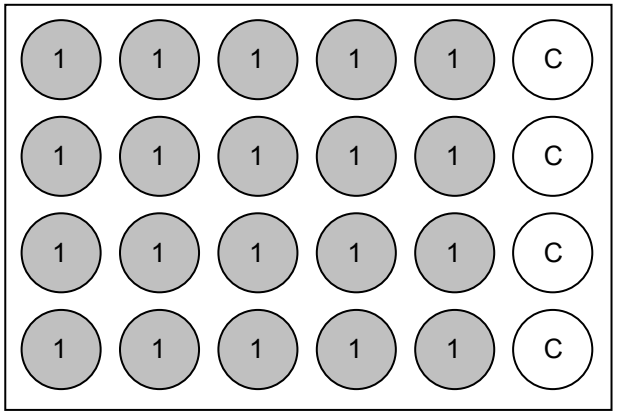
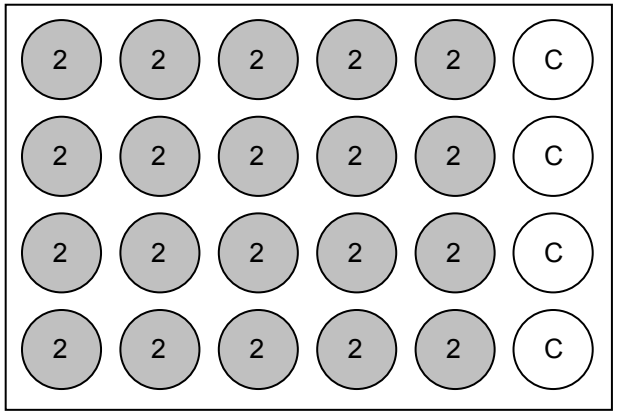
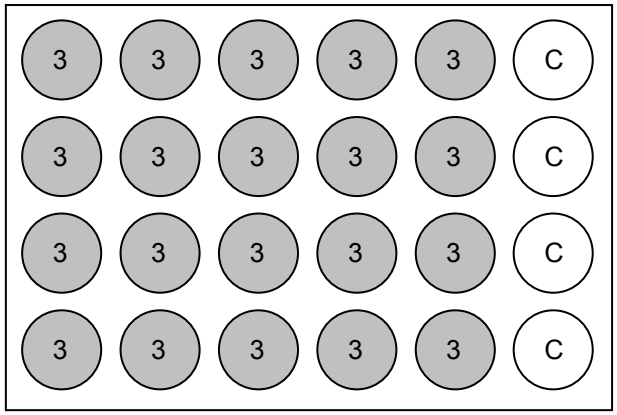
Nagel, R. (2002) DarT: The embryo test with the zebrafish Danio rerio – a general model in ecotoxicology and toxicology. ALTEX 19: 38-48.

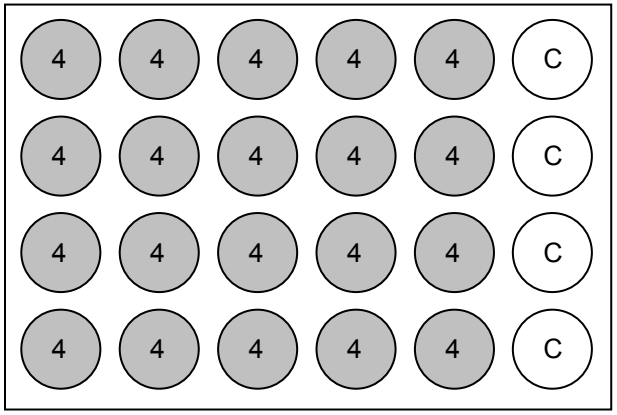
OECD (1992) Test Guideline 203. OECD Guideline for Testing of Chemicals. Fish, Acute Toxicity Test. Available: [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html].

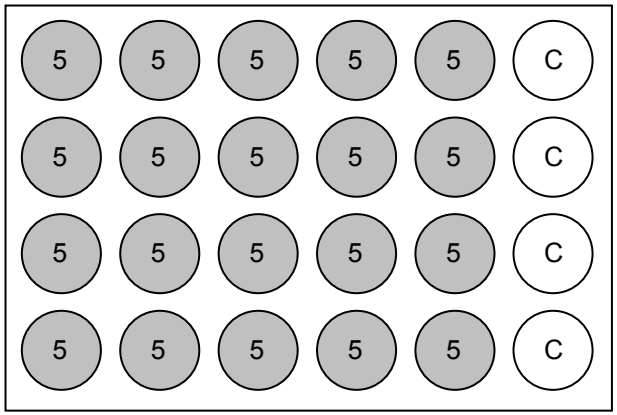
ANNEX 1: Layout of 24-well plates for Phase 1b

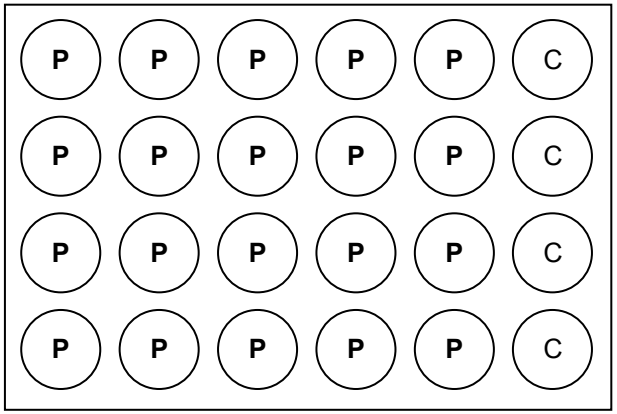


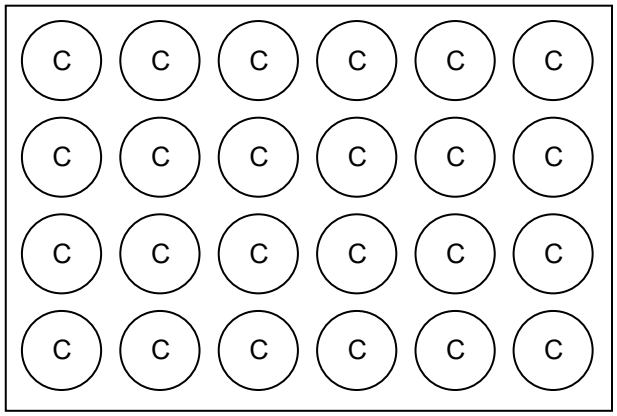


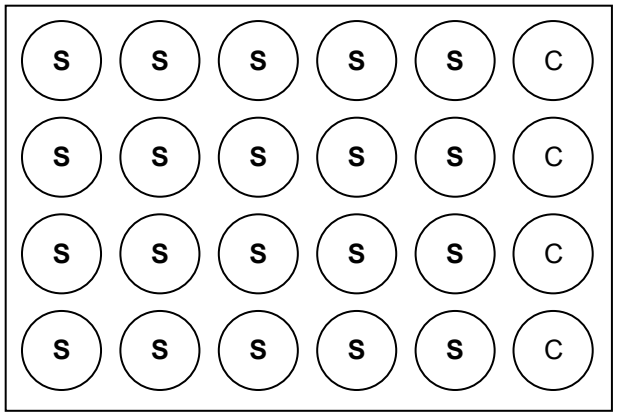












- 1 to 5 = 5 concentrations of the test chemical
- C = negative Controls (dilution water)
- P = Positive controls (3,4 Dichloroaniline)
- S = Solvent controls

ANNEX 2: Statistical analysis

Responsible – Andre Kleensang

As a basis, the following data analyses steps will be performed. Any deviations should be justified and explained in the report of the statistical data analysis. The analyses are not necessarily limited to the given steps.

1. Quality checks

1.1. Is the information complete?
1.2. Are acceptance criteria met?
1.3. Are reported results consistent? (i.e. Is an embryo reported as dead at 24 h still reported as dead at 96 h?)

2. Descriptive statistics

2.1. Summarise quality checks
2.2. Summarise results of chemical and control in tables and figures
2.3. Count failed (e.g. acceptance criteria not met (see 7.1 in SOP) or following the judgement of the operator) and summarise in tables
2.4. Summarise remarks

3. Inferential statistics

3.1. Choose appropriate model for estimating the LC50 including (robust) confidence intervals by following recommendations of the OECD Guidance Document No. 54 on current approaches in the statistical analysis of ecotoxicity data will be considered. As target one model should be chosen which showed an acceptable fit and robustness for all results (exceptions are possible but only for experiments where the chosen model obviously does not show an acceptable fit or the maximization process fails).
3.2. Quality criteria for fitting a model:
- Do the assumptions of the model reflect the biological context?
- Inspection of residuals
- Transformations of the variables are indicated (e.g. log dose)?
- Convergence of maximization process

3.3. Estimate LC50 and confidence intervals per experiment
- Summarise model fits, quality criteria and confidence intervals
- Summarise dose-response curves in figures

3.4. Test of effect on internal controls caused by the increasing test concentrations using Cochran-Armitage trend test in a stratified manner (strata: laboratory).
3.5. Fisher test internal control vs. external control plate in a stratified manner (strata: laboratory).

4. Intralaboratory variability

4.1. Calculate coefficient of variation (CV) based upon LC50 estimates per lab. Will be performed on a log scale if necessary.
4.2. Fitting of a global random effects model on all LC50 estimates, on log scale (if necessary), which simultaneously estimates the components of variability due to within-lab replication, and between lab.

5. Interlaboratory variability

5.1. Calculate CV based upon the LC50 estimate per lab. Will be performed on a log scale if necessary.

5.2. ANOVA and Post-hoc with laboratory (independent variable) vs. LC50 (depended variable).

5.3. See 4.2

6. Report of statistical data analysis

The outcome will be summarised in a report to the validation management group.

7. Quality assurance of data analysis and reporting

An independent statistician (e.g. of IHCP) will review the data analysis and the report.

ANNEX 3:
Sampling of Test Stock Solutions

The Analytical Laboratories of Procter & Gamble will be responsible for the determination of concentrations of four test substances in stock solutions as part of the phase 1b.

Note:

Procter & Gamble will distribute instructions for shipment of sampled solutions to each participating laboratory identified in Section 4 of the Trial Plan for Phase 1b – Testing of six chemicals.

Two samples per stock solution per laboratory are requested. Sample 1 will be the primary sample for analysis and Sample 2 will serve as a back-up in reserve in the case of spillage or other laboratory issue.

1. Labeling Sample Containers:

- Samples should be clearly and legibly labeled with the following information at a minimum:
  - Researcher name
  - Laboratory name
  - Material name and CASNO
  - Nominal concentration of sample
  - Date sample was taken
  - Type of sample (i.e., stock solution)
  - Sample code (consisting of two letter location indicator, date on DDMMYY format followed by -1 or -2 as further described below)
    - An example code from Procter & Gamble's Aquatic Toxicology Laboratory may look like "PG030509-1" for a sample taken by Procter & Gamble on 3 May 2009, sample 1).

2. Sample Containers:
- Use amber borosilicate glass, VWR catalogue 80076-572 or similar (e.g., Wheaton #W224604), with screw caps (solid-top lined with PTFE faced 14B white styrene-butadiene rubber).
- Minimum volume 10 mL, maximum volume 20 mL
- Pre-rinse any sample container with an initial sample
- Fill container completely and cap
- Wrap cap with Parafilm or equivalent
- Wrap entire sample in aluminum foil

3. Packing of samples
   - information will be provided

4. Shipping samples
   - information will be provided

ANNEX 4:
Contact details of laboratories

1st contact	2nd contact
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Zebrafish Embryo Toxicity Test

Standard Operation Procedure

SOP ZFET OECD V02.9 Draft

November 13th, 2009

Zebrafish Embryo Toxicity Test − ZFET

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PAGE OF CHANGES

Date of change	Version number	Changed pages/sections	Summary of the change(s)	Changed by/sign
16/06/2009	V02.8	4, 7, 8, 12, 13	Minor editorial changes	M. Halder on behalf the VMG
		7	5.1: - Vacuum pump was added - Lids to cover 24-well plates were added	M. Halder on behalf the VMG
		8	5.3: - Hardness $CaCO_3$ amended to total hardness	M. Halder on behalf the VMG
		12	6.3.2: - Presaturation of 24-well plates and glass vessels becomes mandatory - Freshly prepared test solutions = prepared on the day of the test	M. Halder on behalf the VMG
		15	New section 6.5 Semi-static renewal procedure added	M. Halder on behalf the VMG
		15	6.6 renamed and revised, i.e. new instructions for measurements of test conditions	M. Halder on behalf the VMG
		16	7.1 Acceptance criteria, see bullet point (6)	M. Halder on behalf the VMG
06/11/09	V02.9	5, 11, 13, 16	Minor editorial changes	M. Halder on behalf the VMG
		8	Table 1 list of chemicals	M. Halder on behalf the VMG
		14, Annex 2	6.4 a minimum magnification 80x is used when scoring heart beat	M. Halder on behalf the VMG
		16	7.1 An acceptance criteria for the positive control (3.4 DCA) is now defined	M. Halder on behalf the VMG

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1 PURPOSE

This Standard Operation Procedure describes a Fish Embryo Toxicity test with the zebrafish (*Danio rerio*; Braunbeck *et al.,* 2005). This test is designed to determine the lethal effects of chemicals on embryonic stages of fish and constitutes an alternative test method to the acute toxicity tests with juvenile and adult fish, i.e., the OECD Test Guideline 203 (OECD TG 203, 1992), thus providing a reduction in fish usage.

2 SCOPE / LIMITATIONS

The method described below is for the evaluation of the Zebrafish Embryo Toxicity test (ZFET), which has been designed as an alternative to the acute fish toxicity test for chemical substances according to OECD TG 203 (OECD TG 203, 1992).

Some substances may cause delayed hatch beyond 96 hours, which will preclude the exposure of eleutheroembryos. In cases, when chemical exposure after hatch seems indispensable, other tests, e.g. OECD TG 203 (OECD TG 203, 1992), might be performed. Known examples of substances requiring prolonged exposure to the eleutheroembryos stage are quaternary ammonium salts.

3 METHOD OUTLINE

Zebrafish embryos are individually exposed in, e.g., 24-well microtiter plates or crystallization dishes. The main criteria for selecting the test vessels should be (a) their inertness (OECD TG 215, 2000) and (b) their volume, since the volume of test solution has to be sufficient for chemical analysis. The test is initiated immediately after fertilization and is continued for 96 hours. Lethal effects, as described by four apical observations (coagulation of the embryo, non-detachment of the tail, non-formation of somites and non-detection of the heart beat), are determined by comparison with controls to identify the LC_{50} value. In addition, non-hatch will be recorded. The test method is based on using a minimum of five test concentrations as well as appropriate negative and positive controls. Each chemical is tested with 20 embryos per test concentration and controls.

4 LIST OF TERMS

4.1 Abbreviations

cm	centimeters
°C	degree Celsius
d	day(s)
DMSO	dimethyl sulfoxide
Fig	figure
g	gram
h (hrs)	hour(s)
ISO	International Organization for Standardization
LC_{50}	test concentration causing 50 % mortality in test organisms
L	liter
m	meter
M	molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
p.a.	per analysis
µl	microliter
OECD	Organisation for Economic Co-operation and Development
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SOP	standard operation procedure
tbd	to be determined
TG	test guideline
ZFET	Zebrafish fish embryo toxicity test
%	per cent

4.2 Definitions

Lethal endpoints	Lethal endpoints indicate acute toxicity to the zebrafish embryo and, consequently, death of the embryos. These are: coagulation of the embryo, non-detachment of the tail, non-formation of somites and non-detection of the heart beat.
Mortality	Observation of one of the above mentioned lethal endpoints indicates mortality.
Survival	Lethal endpoints are not observed.

5 MATERIALS

5.1 Equipment, glass and plastic ware

- Fish maintenance tanks made of chemically inert material and of a suitable capacity in relation to the recommended loading[1];
- pH-meter;
- Oxygen meter;
- Equipment for determination of hardness of water and conductivity;
- Spawn trap:
  - instrument trays of glass, stainless steel or other inert material (e.g., L×W×H = 30 cm × 18 cm × 6 cm);
  - wire mesh of stainless steel or other inert material (e.g. grid size 2 mm) indented about 1 cm into the tray;
  - spawning substrate (e.g., plant imitates of inert material);
- Glass vessels to prepare different test concentrations and dilution water (e.g. beakers, graduated flasks, graduated cylinders, crystallisation dish) or to collect zebrafish embryos (e.g. beakers, crystallisation dish);
- Pipettes;
- Inverted microscope and/or binocular with at minimum 30-fold magnification. If the room cannot be adjusted to 26 ± 1 °C, a temperature-compensated cross movement stage is necessary (e.g. Minitüb HT 200, Tiefenbach, Germany);
- Test chambers; e.g., 24-well exposure plates (e.g. Nunc multidish Nunclon 144530; Renner TPP 92424);
- Self-adhesive foil to cover the 24-well plates (e.g. Nunc Sealing Tape SH, no. 236269) or lids provided with plates if available;
- Incubator or air-conditioned room maintained at 26 ± 1 °C;
- Pasteur pipettes to collect eggs.

[1] cf. section 6.1

5.2 Chemicals

Table 1. List of chemicals

Name [formula]	CAS nº.	Purity	Supplier	Catalog nº.
3,4-Dichloroaniline [$Cl_2C_6H_3NH_2$]	95-76-1	99	Sigma-Aldrich (Fluka Pestanal® analytical standard)	35827
Calcium chloride dehydrate [$CaCl_2 \cdot 2\ H_2O$]	10035-04-8	p.a.	e.g., Merck	1.02382.0500
Magnesium sulfate heptahydrate [$MgSO_4 \cdot 7\ H_2O$]	10034-99-8	p.a.	e.g., Merck	1.05886.0500
Sodium carbonate [$NaHCO_3$]	144-55-8	p.a.	e.g., Merck	1.06329.0500
Potassium chloride [KCl]	7447-40-7	p.a.	e.g., Merck	1.04936.0500
Hydrochloric acid [HCl]	7647-01-0	p.a.	e.g., Merck	1.09063.1000
Sodium hydroxyde [NaOH]	1310-73-2	p.a.	e.g., Merck	1.09136.1000

5.3 Dilution water for the zebrafish embryo toxicity test

For the zebrafish embryo toxicity test (ZFET), dilution water is prepared according to OECD TG 203 Annex 2 (1992):

- 294.0 mg/L $CaCl_2 \cdot 2\ H_2O$;
- 123.3 mg/L $MgSO_4 \cdot 7\ H_2O$;
- 64.7 mg/L $NaHCO_3$;
- 5.7 mg/L KCl.

The resulting degree of total hardness should be equivalent to 10 - 250 mg/L. The water is aerated until oxygen saturation is achieved, then stored for about two days without further aeration before use. The pH should be adjusted to a range between pH 6.5 and 8.5. Use of HCl and NaOH is recommended. The conductivity of the distilled or deionized water used for preparing the dilution water should not excess 10 µS/cm.
Dilution water temperature should be 26.0 ± 1.0 °C when used for preparation of test concentrations/controls.

Table 2: Preparation of dilution water

Stock solution	Compound		Distilled or deionized water volume	Add	Final volume
1	$CaCl_2$ 2 H_2O	14.700 g	500 ml	10 ml	1 L
2	$MgSO_4 \cdot 7\ H_2O$	6.165 g	500 ml	10 ml	
3	$NaHCO_3$	3.235 g	500 ml	10 ml	
4	KCl	0.285 g	500 ml	10 ml	

Stock solutions are 100fold concentrated in comparison to concentrations finally used in the test; therefore solutions must be diluted by the factor 100. For 1 liter dilution water in the fish embryo test, 10 ml of each stock solution are required.

6 METHODS

6.1 Maintenance of zebrafish broodstock

A breeding stock of unexposed, mature zebrafish with an age between 4 and 18 months is used for egg production. Each laboratory should precisely specify strain, origin of the strain, duration of maintenance in the particular laboratory and reproductive performance (fecundity, standard fertilization rate). In any case, on a regular basis (at least, each 6 months), the LC_{50} of the standard positive control 3,4-dichloroaniline ($Cl_2C_6H_3NH_2$; CAS 95-76-1; Fluka Pestanal® analytical standard, Sigma-Aldrich no. 35827) should be determined[2], and the LC_{50} should range between 1.6 and 4.4 mg/L after 48 hpf (Braunbeck *et al.*, 2005; Lange *et al.*, 1995; Schulte, 1997).

Fish should be free of macroscopically discernable symptoms of infection or disease and should not have been treated with any pharmaceutical (acute or prophylactic) treatment for 2 months before spawning. Spawners are maintained in aquaria with a loading capacity of a minimum of 1 L water per fish and a fixed 12-16 hour light photoperiod (Braunbeck *et al.*, 2005; Nagel, 2002; Schulte and Nagel, 1994; Laale, 1977; Westerfield, 2000). Males and females are continuously held together. Oxygen saturation ≥ 80 % should always be maintained for keeping and breeding; water temperature should be adjusted to 26 ± 1 °C. Optimal filtering rates should be adjusted; excess filtering rates causing heavy perturbation of the water should be avoided. Alternatively, permanent flow-through or semi-static conditions may be used to guarantee that ammonia, nitrite, and nitrate levels are kept below the critical limit for toxicity (0 - 5, 0.025 - 1 and 0 - 140 mg/L, respectively). Fish are fed with commercially available artificial diets (e.g., TetraMin™ flakes; Tetra, Melle, Germany) at regular intervals (e.g. 3 to 5 times daily would be optimal), occasionally supplemented with brine shrimp (*Artemia* spec.) nauplii or small daphnids of appropriate size obtained from an uncontaminated source. Overfeeding should be strictly avoided to ensure optimal water quality; remaining food and feces should be removed daily. From three days before spawning, feeding with brine shrimp (*Artemia* spec.) twice daily (*ad libitum*) is recommended to achieve optimal mating.

6.2 Egg production

6.2.1 Background

Under spawning conditions, male zebrafish can easily be distinguished from females by their more slender body shape and an orange to reddish tint in the silvery bands along the body. Due to the

[2] Not applicable for phase 1b

large number of eggs produced, females can be recognized by their swollen bellies (Fig. 1). Egg production can be performed via spawning groups (6.2.2) or mass spawning (6.2.3).

A single mature female spawns at least 50 - 80 eggs per day. Depending on the strain, spawning rates may be considerably higher. The fertilization rate should be ≥70 %. In case first time spawning fish are used, fertilization rates may be lower in the first few spawns.

Fig. 1: Sexually mature zebrafish (*Danio rerio*). Adult zebrafish females (upper individual) can easily be differentiated from males (lower individual) by their extended bellies and the lack of reddish tint along the silvery longitudinal lines. Photo: Erik Leist, Heidelberg.

6.2.2 Egg production *via* spawning groups

Note: Annex 3 describes egg production in spawning groups as used at the University of Heidelberg (Germany). A more general description is given in the following.

The day before a test, males and females are placed in spawning tanks a few hours before the onset of darkness. Since spawning groups of zebrafish may occasionally fail to spawn, the parallel use of at least three spawning tanks is strongly recommended.

For collection of eggs, trays covered with a grid are placed into the spawning tanks before the onset of darkness. If considered necessary, artificial plants made of green plastic or glass can be fixed to the grid as spawning stimulus. Mating, spawning and fertilization take place within 30 min after the onset of light in the morning and the egg trays can be carefully removed.

For selection of fertilized eggs see 6.3.2.1.

6.2.3 Egg production *via* mass spawning

Alternatively, eggs may be collected with larger trays covered with a grid. They are placed at the bottom of the normal maintenance tanks before the onset of darkness. If considered necessary, artificial plants made of green plastic or glass can be fixed to the grid as spawning stimulus. Mating, spawning and fertilization take place within 30 min after the onset of light in the morning and the egg trays can be carefully removed.

For selection of fertilized eggs see 6.3.2.1.

6.3 Zebrafish Embryo Toxicity Test

6.3.1 Test concentrations and controls

NOTE: The following describes the general procedures. ***For the validation study, the procedures given in the trial plan should be followed, e.g. preparation of stock solutions, test concentrations, use of solvent, negative and positive controls, number of runs****.*

Chemicals should be tested in 5 concentrations spaced by a constant factor not exceeding 2.2 and prepared as dilutions with standard dilution water (see 5.3). Test solutions of the selected concentrations can be prepared, e.g., by dilution of a stock solution. The stock solutions should preferably be prepared by simply mixing or agitating the test substance in the dilution water by mechanical means (e.g., stirring or ultrasonification). If the test substance is difficult to dissolve in water, procedures described in the OECD Guidance Document No. 23 for handling difficult substances should be followed (OECD GD 23, 2000). The use of solvents or dispersants (solubilizing agents) should, if ever possible, be avoided, but may be required in some cases in order to produce a suitably concentrated stock solution. Additionally to the examples of suitable solvents given in OECD (OECD TG 215, 2000), dimethyl sulfoxide (DMSO) might be useful. In case a solubilizing agent is required to assist in stock solution preparation, its final concentration should not exceed 1000 µl/L for most of the commonly used solvents. The solvent concentration should be the same in all test vessels. In case a solvent has to be used, a separate solvent control has to be run (see trial plan).

Justification should be provided if fewer than five concentrations are used. The highest concentration tested should preferably result in 100% mortality, and the lowest concentration tested should preferably give no observable effect. A range-finding test properly conducted before the definitive test enables the choice of the appropriate concentration range.

Pure dilution water is used as a negative control. Negative controls are required both as internal and as external controls. For localization of negative controls, see trial plan.

As a positive control, 3,4-dichloroaniline should be tested at a concentration of **4 mg/l.**

Each chemical is tested with 20 eggs/embryos per test concentration and controls.

There should be evidence that the concentration of the substance being tested has been satisfactorily maintained, and preferably it should be at least 80 % of the nominal concentration throughout the test[3]. If the deviation from the nominal concentration is higher than 20 %, results should be based on the measured concentration.

[3] Not applicable in Phase 1b, test concentrations will be only measured in one laboratory.

6.3.2 Exposure of fish embryos

NOTE: The 24-well plates and glass vessels must be pre-saturated with the respective concentrations of test substances and controls for <u>at least</u> 24 hrs before the day of the test. Glass vessels and 24-well plates are filled with the required quantity of freshly prepared test concentrations (freshly = prepared on the same day) and respective controls (see 6.3.2.1; 6.3.2.2 and trial plan).

In order to start exposure with minimum delay, at least twice of the number of eggs needed per treatment group (see 6.3.2.2) are randomly selected and transferred not later than 1 h post fertilization, into glass vessels containing an appropriate volume (e.g. 50 ml; eggs should be fully covered) of the different test concentrations and respective controls. Viable fertilized eggs should be separated from unfertilized eggs (see 6.3.2.1) and be transferred to 24-well plates within 3 h post fertilization (Fig. 2).

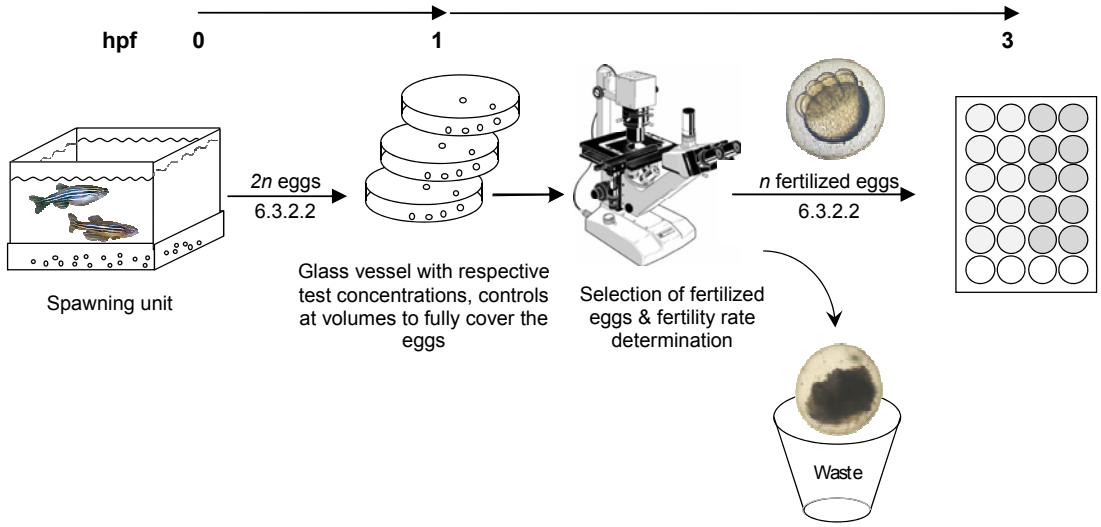

Fig. 2: Scheme of the ZFET test procedure (from left to right): collection of the eggs, pre-exposure immediately after fertilization in glass vessels, selection of fertilized eggs with an inverted microscope or binocular and distribution of fertilized eggs into prepared 24-well plates, n = number of eggs required for the test run.

6.3.2.1 Selection of fertilized eggs

The glass vessels containing the eggs (as described in 6.3.2.) are placed under an inverted microscope or a binocular with a minimum magnification of 25× to identify fertilized eggs and determine the fertility rate. Fertilized eggs can easily be identified by their transparency (see Fig 3), at best by putting the glass vessels on a black pad and using flexible swan neck lights or transverse light under the binocular.

In the following, details on the appearance of developmental stages critical for the identification of fertilized eggs are given (see also Annex 1):

- Freshly spawned eggs are characterized by a fully transparent perivitelline space surrounded by the egg membrane and containing the yolk, and the germinal disc, which has already formed at the animal pole.
- After fertilization, the first cell division is initiated at 26 °C after about 15 min.
- From the 4-cell stage onwards, fertilized eggs can unambiguously be distinguished by their transparency from non-fertilized eggs.

- Eggs with overt anomalies (asymmetries, formation of vesicles) or damaged membranes should be discarded.
- Non-fertilized eggs can be identified by a lack of blastomer formation and, at later stages, by their non-transparency.

NOTE: For the ZFET, only fertilized eggs between the 4- and 128-cell stages should be used.

Fig. 3: Batch of newly spawned zebrafish (*Danio rerio*) eggs. Photo: Dr. T. Meinelt, Institute of Freshwater Ecology and Inland Fisheries, Berlin, FRG.

6.3.2.2 Distribution of eggs over 24-well plates

Fertilized eggs are individually transferred to the freshly prepared 24-well plates (final volume of 2 ml per well) and distributed as given in the trial plan (TP_ZFET_OECD_1b_V01.1):

- 20 eggs for each of the test concentrations on a separate plate;
- 4 eggs as negative internal control per plate;
- 20 eggs as negative external controls on a separate plate;
- 20 eggs for the solvent control on a separate plate;
- 20 eggs for the positive control on a separate plate.

6.3.2.3 Incubation conditions

The 24-well plates are covered with self-adhesive foil or lids provided with plates and incubated at 26 ± 1 °C for 96 hrs. Control of the light cycle to 14 h light and 10 h dark is achieved by keeping the eggs in either an incubator or separate room equipped with an automatic light control.

6.4 Determination of chemical toxicity (toxicological endpoints)

The following four endpoints indicate acute toxicity and, consequently, death of the embryos:

- coagulation of the embryo,
- non-detachment of the tail,
- non-formation of somites and
- non-detection of the heart beat.

These lethal endpoints are recorded after 24, 48, 72 and 96 hrs as listed in Table 3.

NOTE: Observation of <u>one</u> of the above mentioned lethal endpoints indicates mortality.

Table 3: Lethal endpoints and their recording in the Zebrafish Embryo Toxicity Test (ZFET)

	Exposure time (h)			
	24	48	72	96
Coagulated embryos	+	+	+	+
Tail not detached	+	+	+	+
No somite formation	+	+	+	+
No heart beat		+	+	+

Coagulation of the embryo: Coagulated embryos are milky white and appear dark under the microscope (See Annex 2, Fig. A2a). The number of coagulated embryos is determined after 24, 48, 72 and 96 hrs.

Tail not detached: In a normal developing zebrafish embryo, detachment of the tail (see Annex 2, Fig. A2b) from the yolk is observed following posterior elongation of the embryonic body. Absence of tail detachment is recorded after 24, 48, 72 and 96 hrs.

No somite formation: At 26 ± 1°C, about 20 somites have formed after 24 hours (see Annex 2, Fig. A2c) in a normal developing zebrafish embryo; however, it is not possible to determine the exact number at this time (spontaneous movements indicate the formation of somites). A normally developed embryo shows spontaneous movements (side-to-side contractions). The absence of somites is recorded after 24, 48, 72 and 96 hrs.

No heart beat: In a normal developing zebrafish embryo at 26 ± 1 °C, the heart beat is visible after 48 hrs (see Annex 2, cf. Fig. A2d). Absence of heart beat is recorded after 48, 72 and 96 hrs. Particular care should be taken when recording this endpoint, since irregular (erratic) heart-beat should *not* be recorded as lethal. Moreover, visible heart beat without circulation in aorta abdominalis is considered non-lethal. The observation time to record an absence of heart beat should be at least of 1 min with a minimum magnification of 80×.

Hatching rate and post-hatch mortality: Since zebrafish embryos usually hatch after 72 hrs, non-hatching may represent an important toxic effect. However, since the time to hatch may differ between test concentrations, controls may have already hatched, whereas embryos exposed to the test concentration may still have not. Hatching rates will not be used for the calculation of LC_{50} values. In case of abnormal hatching time, hatching rates should be recorded until 96 hrs. Post-hatch mortality will be covered by observing the above defined lethal endpoints until 96 hours.

NOTE: In addition to the lethal endpoints, other observations should be recorded in the reporting template under "remarks".

6.5 Semi-static renewal procedure

*NOTE: The following steps are carried out **after** the daily recording of the lethal effects (see 6.4)*

Renewal of the test solutions **and** the controls must be performed after 24, 48, 72 hours:

- Test concentrations are freshly prepared from the stock solution (see *NOTE* 6.3.2)
- Solutions are removed by using an appropriate pipette or vacuum suction (cell culture-fitted vacuum pump plus suction bottle). For the removal of *each* test concentration, separate pipette tips must be used.

NOTE: In any case, contact with the eggs must be avoided!

- At least 90% of the volume of each well must be removed and immediately replaced with the corresponding volume of freshly prepared test solutions/controls.

6.6 Measurements of test conditions

Measurements of test conditions should be performed at least on the following time points:

- 0 hour
- 24 hours (old solution)
- 72 hours (fresh renewal solution)
- 96 hours

for the controls and the highest concentration.

The following parameters should be measured by using microprobes or carefully pooling test solutions (e.g., by pipetting):

- The dissolved oxygen concentration should be in compliance with the test requirements (see 7.1).
- The pH should normally be within a range of pH 6.5 and 8.5.
- The total hardness should be within 10 to 250 mg/l (OECD TG 203, 1992).
- The temperature and the conductivity

If the equipment is available, the light intensity can be measured at least once.

The results must be recorded in the corresponding section of reporting template (see 7.2*)*.

7 DATA ANALYSIS AND REPORTING

7.1 Acceptance Criteria

For a test to be considered to fulfill the performance requirements, the following conditions should apply:

(1) The fertility rate of the parent generation should be ≥ 70%.
(2) The dissolved oxygen concentration should be ≥ 80 % of the air saturation value at the beginning of the test.
(3) The water temperature should be maintained at 26 ± 1 °C in test chambers at any time during the test.
(4) Overall survival of embryos in the negative external control and, where relevant, in the solvent control should be ≥ 90% until the end of exposure.
(5) Exposure to the positive control (e.g., 4.0 mg/l 3,4-dichloroaniline) should result in a minimum mortality of 30 %
(6) Test solutions must be renewed on a daily basis (see 6.5).

Note: If acceptance criteria are not met, the test is considered to be failed and needs to be repeated.

7.2 Reporting

A template for reporting will be provided for each phase of the study by the study coordinator: *RT_ZFET_OECD_1b_V01.1_Laboratory code_Chemical_Run*

Each laboratory should use the template to report the results of valid and failed experiments. The file should be returned to the study coordinator. Printed and signed originals should be archived by the laboratories.

7.3 Statistical analysis

The LC_{50} determination and statistical evaluation will be carried out by André Kleensang.

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– ANNEXES –

ANNEX 1:

Table and atlas of normal zebrafish development

ANNEX 2:

Atlas of lethal endpoints for the Zebrafish Embryo Toxicity Test

ANNEX 3:

Egg production in spawning groups as performed at University of Heidelberg

ANNEX 1

Table and atlas of normal zebrafish development

Freshly spawned eggs are characterized by a fully transparent perivitelline space surrounded by the egg membrane and containing the yolk, and the germinal disc, which has already formed at the animal pole. After fertilization, the first cell division is initiated at 26 °C after about 15 min. Subsequently, the germinal disc is divided synchronously into 4, 8, 16 and 32 blastomers after 1 h, 1,25 h, 1,5 h and 1,75 h (Table A1; Figs. A1a, A1c; Kimmel *et al*., 1995). From the 4-cell stage onwards, fertilized eggs can unambiguously be distinguished by their transparency from non-fertilized eggs. For the ZFET, only fertilized eggs between the 4- and 128-cell stages should be used. Eggs with overt anomalies (asymmetries, formation of vesicles) or damaged membranes should be discarded. Non-fertilized eggs can be identified by a lack of blastomer formation and, at later stages, by their non-transparency.

Table A1: Stages of embryonic development of zebrafish (*Danio rerio*) at 26 ± 1 °C (Nagel, 2002)

Time (h)	**Stage**	**Characterization (after Kimmel *et al.*, 1995)**
0	Fertilization	Zygote
0	Zygote period	Cytoplasm accumulates at the animal pole, one-cell stage
0.75	Cleavage period	Discoidal partial cleavage:
		1. (median vertical) division: two-cell-stage
1		2. (vertical) division: four-cell-stage
1.25		3. (vertical and parallel to the plane of the first) division: 8-cell-stage
1.5		4. (vertical and parallel to the second) division: 16-cell-stage
2	Blastula period	Start of blastula stage
3		Late cleavage; blastodisc contains approximately 256 blastomers
4		Flat interface between blastoderm and yolk
5.25	Gastrula period	50 % of epibolic movements; blastoderm thins and interface between periblast and blastoderm become curved
8		75 % of epibolic movement
10		Epibolic movement ends, blastopore is nearly closed
10.5	Segmentation period	First somite furrow
12		Somites are developed, undifferentiated mesodermal component of the early trunk, tail segmented or metameric
20		Muscular twitches; sacculus; tail well extended
22		Side to side flexures; otoliths
24	Pharyngula period	Phylotypic stage, spontaneous movements, tail is detached from the yolk; early pigmentation
30		Reduced spontaneous movement; retina pigmented, cellular degeneration of the tail end; circulation in the aortic arch 1 visible
36		Tail pigmentation; strong circulation; single aortic arch pair, early motility; heart beating
72 - 96	Hatching period	Heart-beat regular; yolk extension beginning to taper; dorsal and ventral pigmentation stripes meet at tail; segmental blood vessels detectable: thickened sacculus with two chambers visible; foregut development; neuromasts

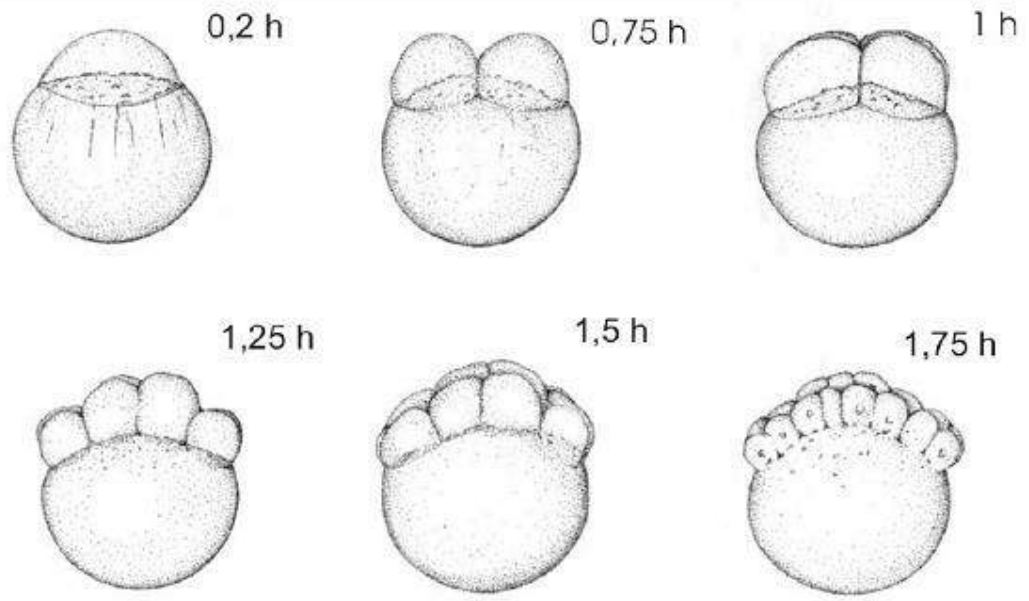

Fig. A1a: **Selected stages of early zebrafish (*Danio rerio*) development**: 0.2 – 1.75 h post-fertilization (from Kimmel *et al.*, 1995). The time sequence of normal development may be taken to diagnose both fertilization and viability of eggs (see paragraph 6.3.2.1: Selection of fertilized eggs).

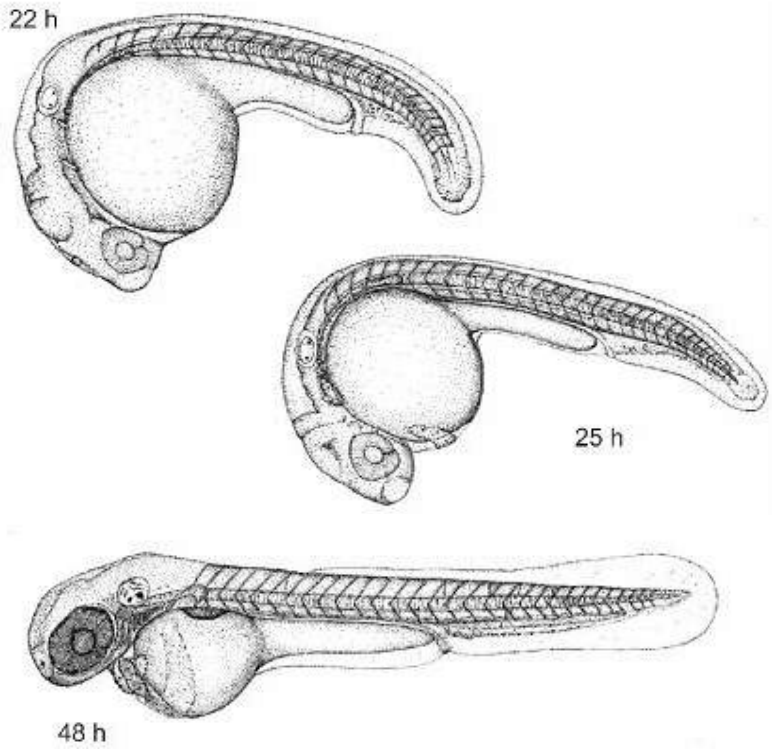

Fig. A1b: **Selected stages of late zebrafish (Danio rerio) development**: 22 - 48 h after fertilization (from Kimmel *et al.*, 1995).

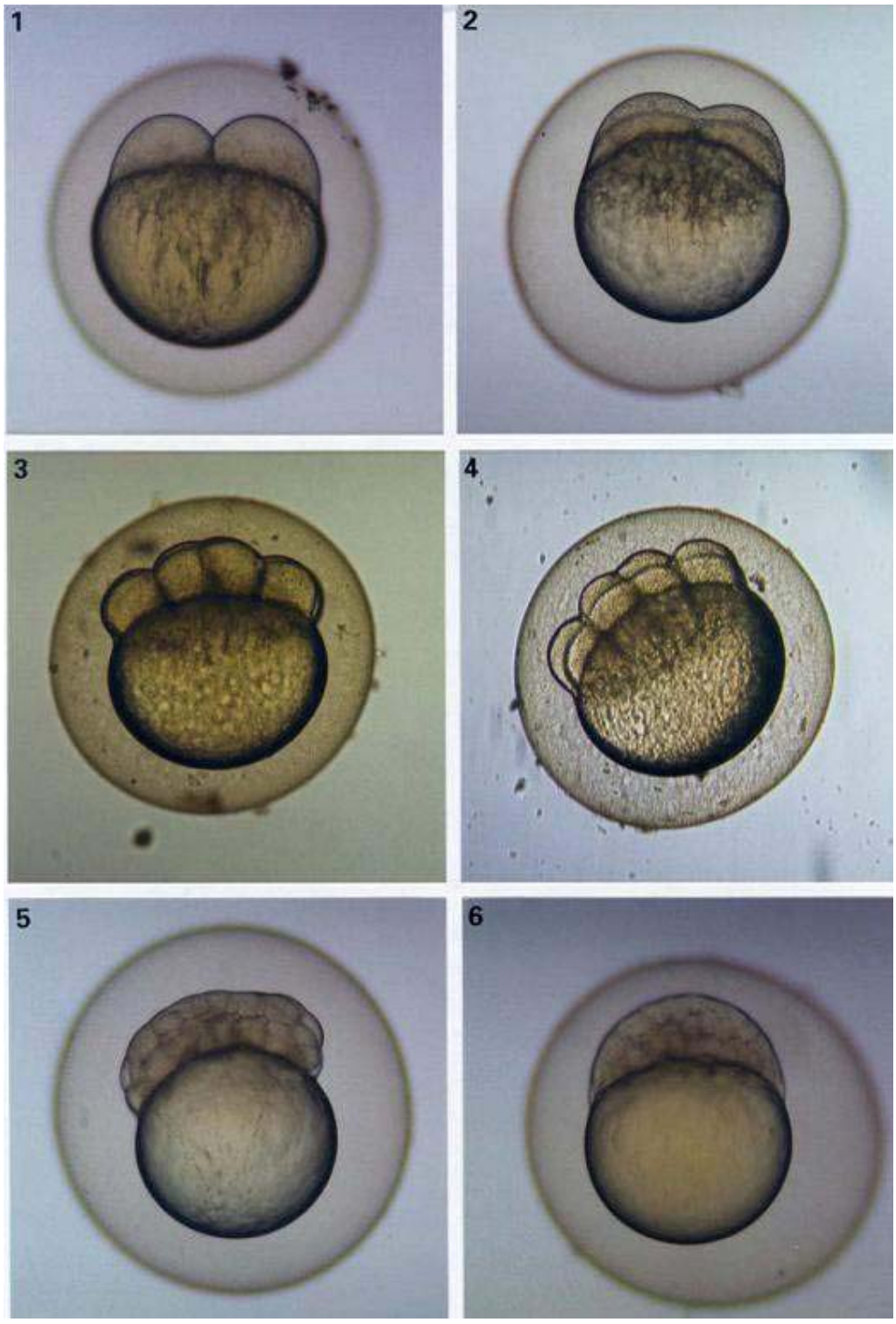

Fig. A1c: **Normal development of zebrafish (*Danio rerio*) embryos** I: (1) 0.75 h, 2-cell stage; (2) 1 h, 4-cell stage; (3) 1.2 h, 8-cell stage; (4) 1.5 h, 16-cell stage; (5) 4.7 h, beginning epiboly; (6) 5.3 h, approx. 50 % epiboly (from Braunbeck & Lammer 2005).

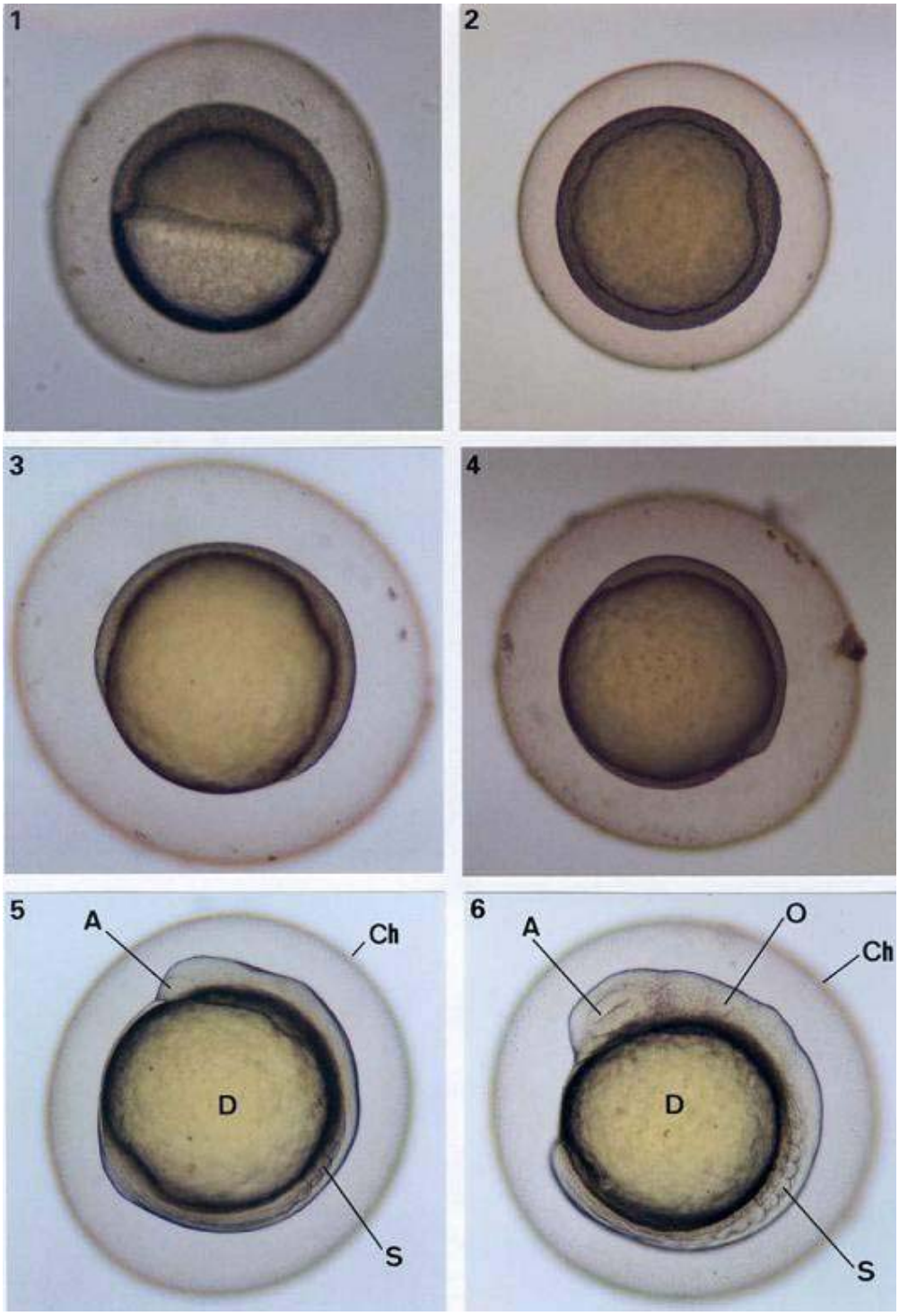

Fig. A1d: **Normal development of zebrafish (*Danio rerio*) embryos** II: (1) 6 h; (2) 6 h; (3) 8 h; (4) 9 h; (5) 12 h; (6) 14 h. A – eye bud; Ch – chorion; D – yolk; O – ear bud; S – somites (muscle segments; from Braunbeck & Lammer 2005).

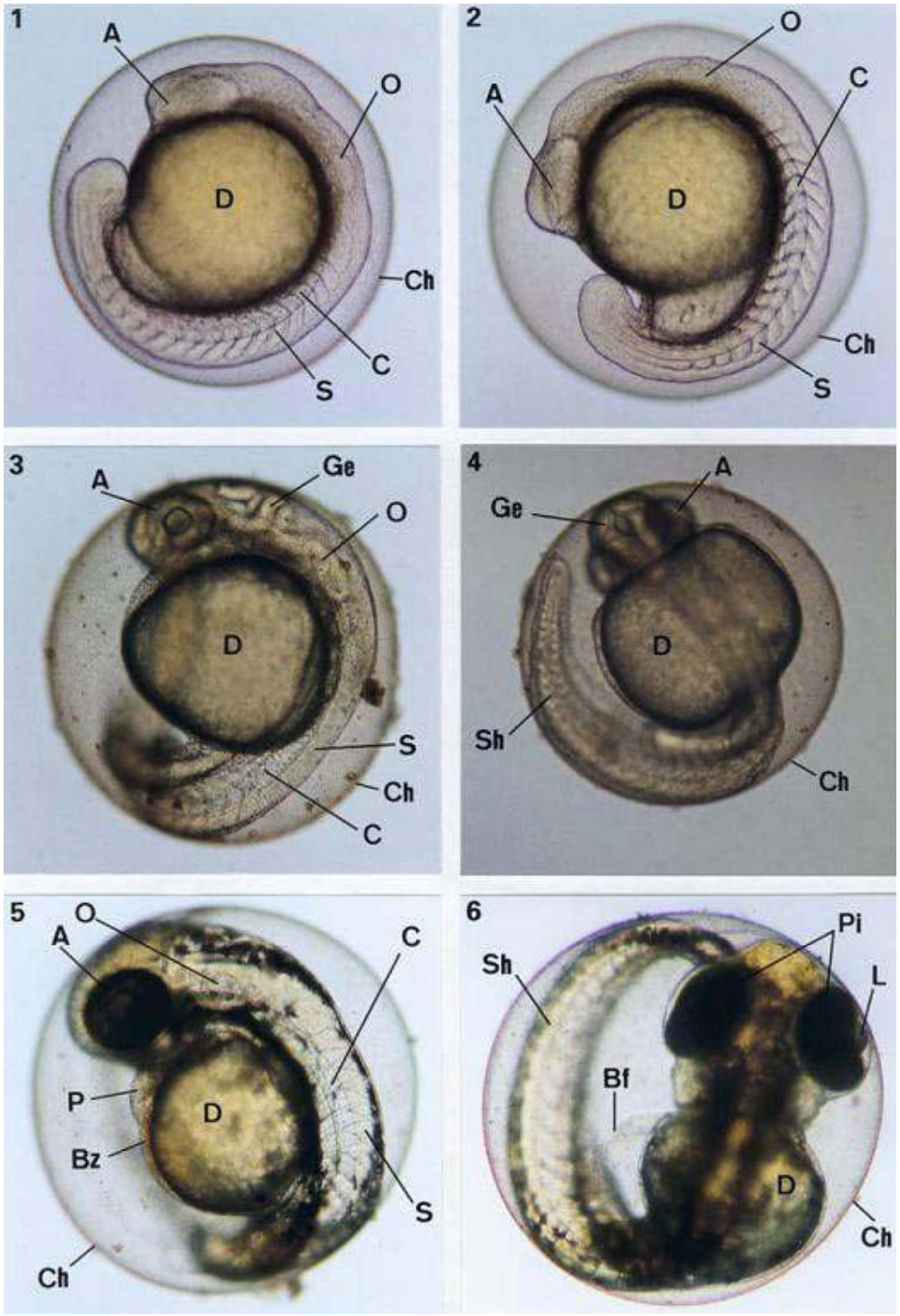

Fig. A1e: **Normal development of zebrafish (*Danio rerio*) embryos** III: (1) 16 h; (2) 18 h; (3) 25 h; (4) 25 h; (5) 48 h; (6) 72 h. A – eye bud; Bf – pectoral fin; Bz – blood cells; C – chorda; Ch – chorion; D – yolk; Ge – brain; L – lens; P – pericardium; Pi – ocular pigment layer; S – Somites; Sh – tail; O – ear bud (from Braunbeck & Lammer 2005).

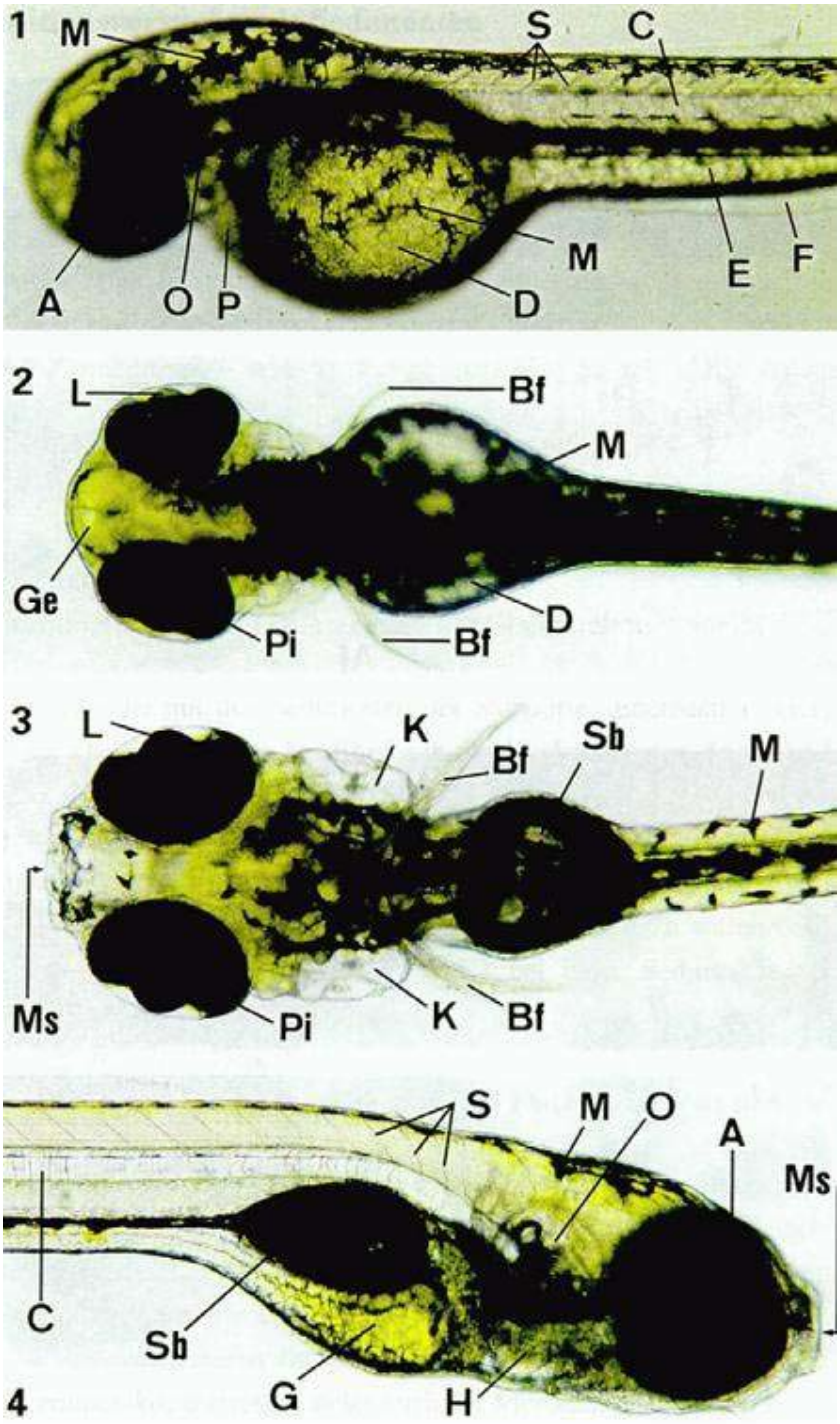

Fig. A1f: **Normal development of zebrafish (*Danio rerio*) embryos** IV: (1) 48 h; (2) 72 h; (3) 144 h; (4) 144 h. A – eye bud; Bf – pectoral fin; C – chorda; D – yolk sac; E – gut; F – fin; G – gastrointestinal tract, Ge – brain; H – heart; K – gills; L – eye lens; M – melanophores; Ms – mouth slit; O – ear; P – pericardium; Pi – ocular pigment layer; S – somites (muscle segments); Sb – swimming bladder (from Braunbeck & Lammer 2005).

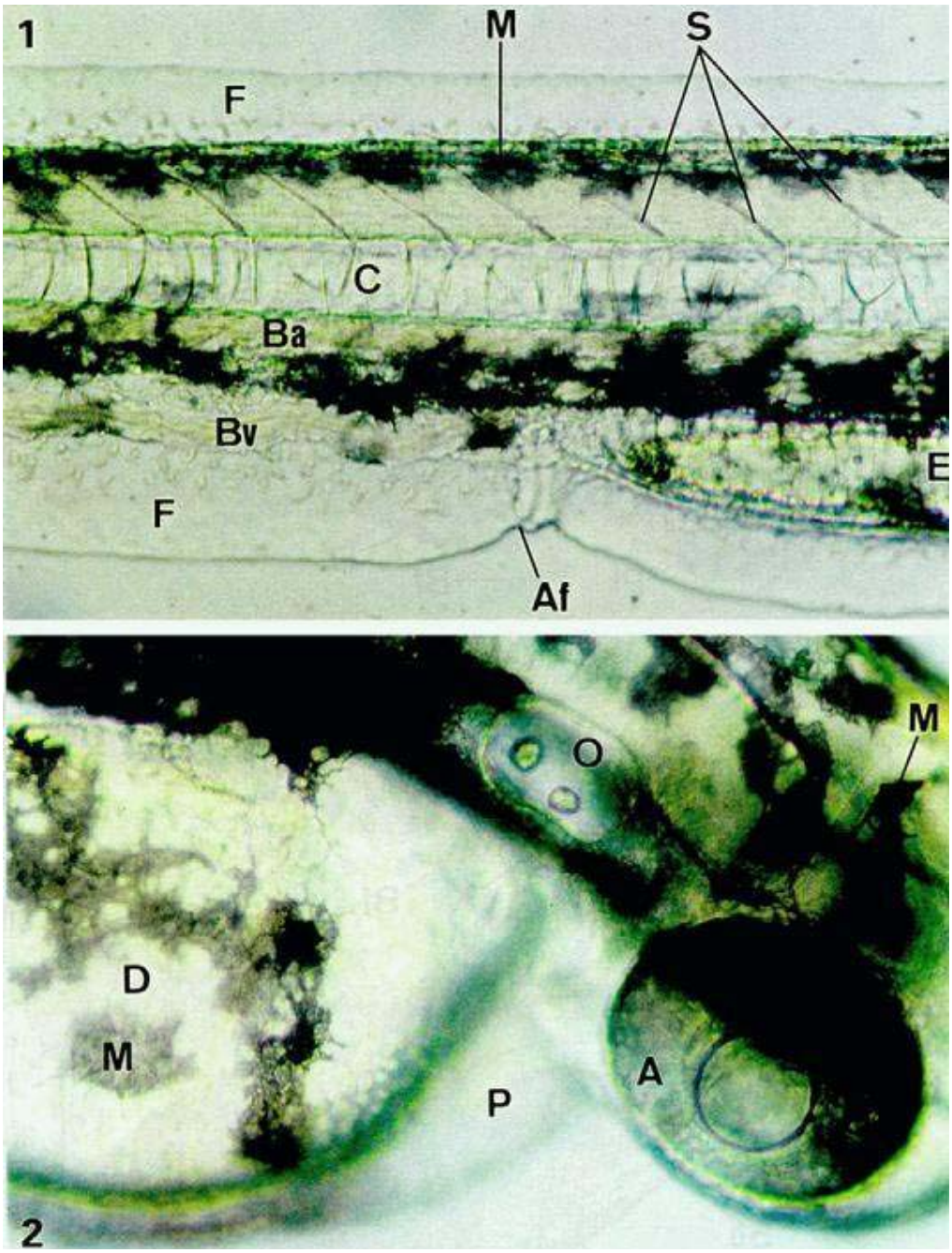

Fig. A1g: **Normal development of zebrafish (*Danio rerio*) embryos** V (following dechorionation): (1) 48 h, anal region; (2) 48 h, ear region. A – eye bud; Af – anus; Ba – dorsal aorta; Bv – central ventral axial vein; C – chorda; D – yolk sac; E – peritoneum; F – fin; M – melanophores; P – pericardium; O – ear; S – somites (muscle segments; from Braunbeck & Lammer 2005).

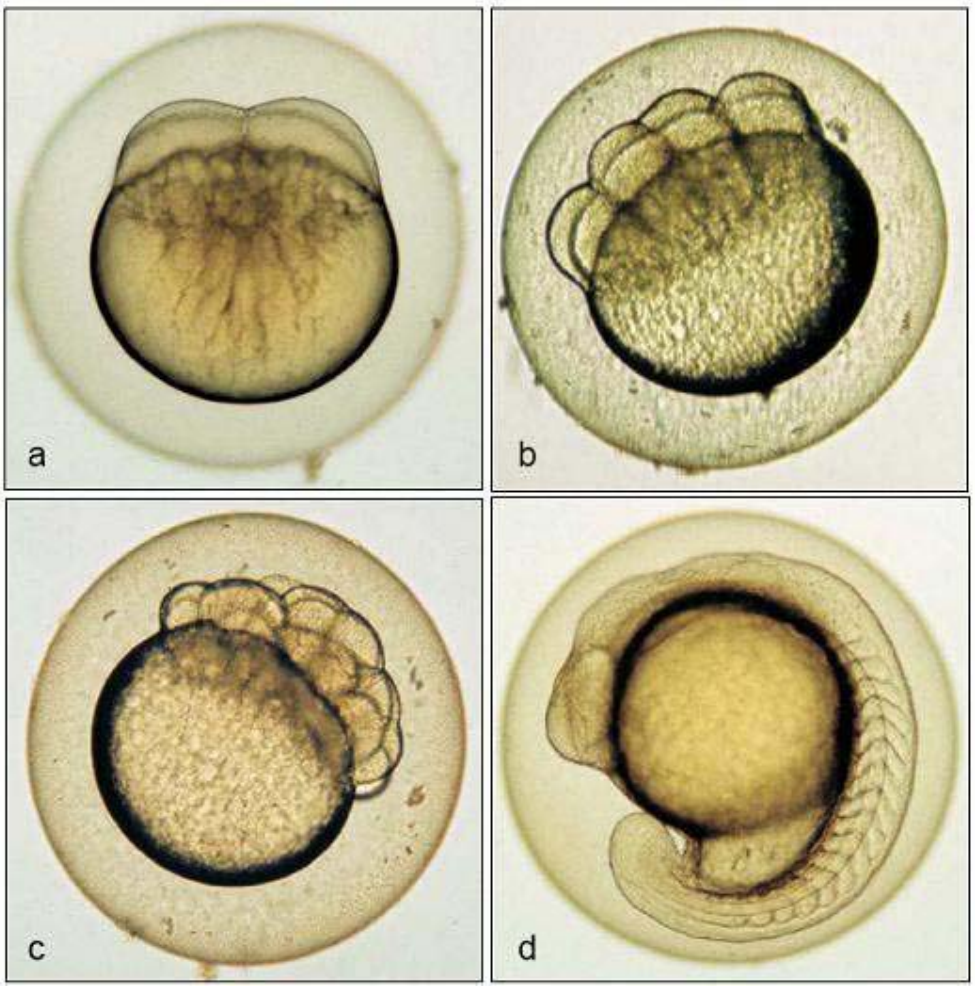

Fig. A1h: **Selected stages of zebrafish (*Danio rerio*) development**: (a) 4-cell stage (approx. 1 h); (b) 16-cell stage (approx. 1.3 h); (c) 64-cell stage (approx. 1.8 h); (d) detachment of tail (approx. 17.5 h; from Braunbeck & Lammer 2005).

ANNEX 2

Atlas of lethal endpoints for the Zebrafish Embryo Toxicity Test

The following apical endpoints indicate acute toxicity and, consequently, death of the embryos: coagulation of the embryo, non-detachment of the tail, non-formation of somites and non-detection of the heartbeat. The following micrographs have been selected to illustrate these endpoints.

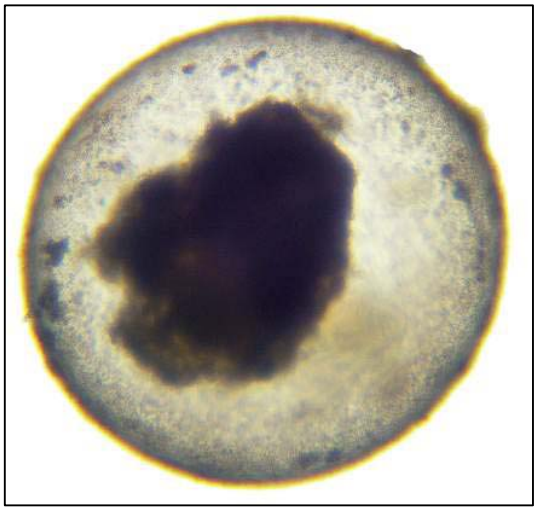

Fig. A2a: **Coagulation of the embryo**: Under bright field illumination, coagulated zebrafish embryo show a variety of intransparent inclusions.

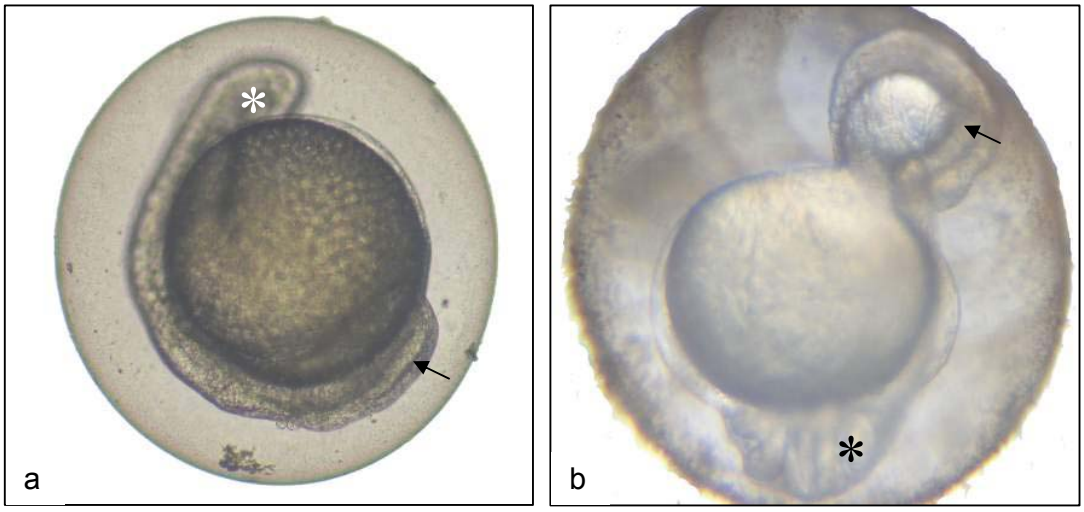

Fig. A2b: **Non-detachment of tail** bud in lateral view (a: →; 96 h old zebrafish embryo) and frontal rear view (b: →; 96 h old zebrafish embryo). Note also the lack of the eye bud (*).

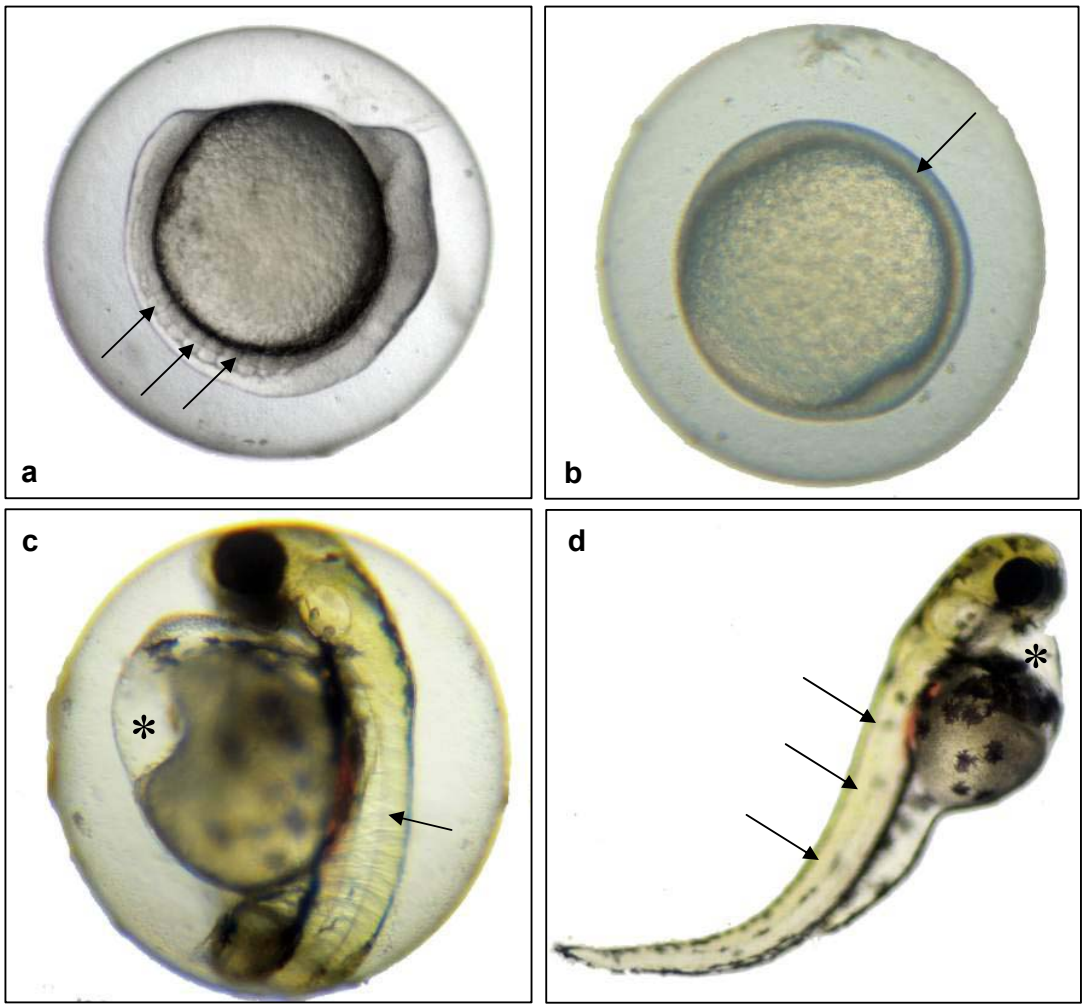

Fig. A2c: **Non-formation of somites**: Although retarded in development by approx. 10 h, the 24 h old zebrafish embryo in (a) shows well-developed somites (a: →), whereas the embryo in the right micrograph does not show any sign of somite formation (b: →). Although showing a pronounced yolk sac edema (*), the 48 h old zebrafish embryo in (c) shows distinct formation of somites (→), whereas the 96 h (!) old zebrafish embryo depicted in (d) does not show any sign of somite formation (→). Note also the spinal curvature (scoliosis) and the pericardial edema in the embryo shown in Fig. (d), see also figure A2d.

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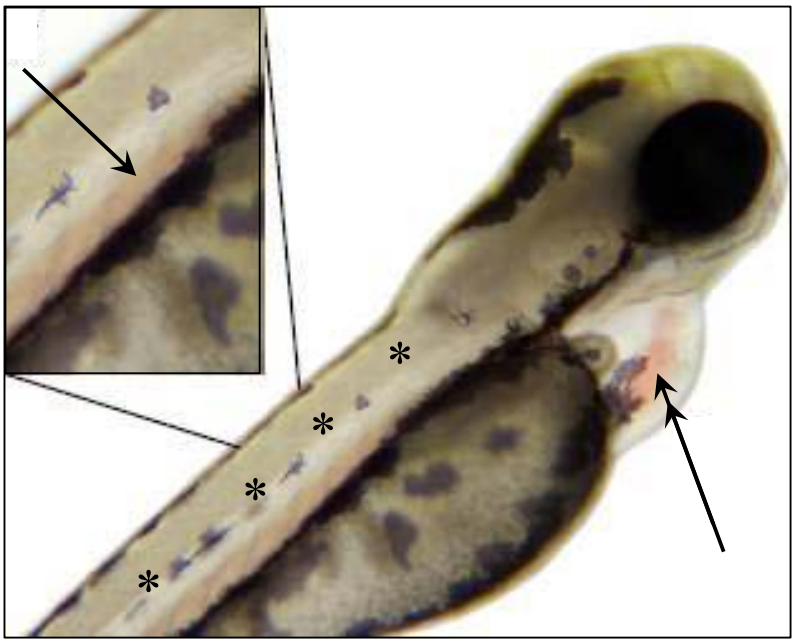

Fig. A2d: **Lack of heart beat** is, by definition, difficult to illustrate in a micrograph. Lack of heart beat is indicated by either non-convulsion of the heart (double arrow) or immobility of blood cells in, e.g., the aorta abdominalis (→ in insert). Note also the lack of somite formation in this embryo (*, homogenous rather than segmental appearance of muscular tissues). The observation time to record an absence of heart beat should be at least of 1 min with a minimum magnification of 80×.

ANNEX 3

Egg production in spawning groups as performed at University of Heidelberg

The day before a test, males and females in a ratio of 2:1 are placed in spawning tanks (Fig. 1) immediately before the onset of darkness. Since spawning groups of zebrafish may occasionally fail to spawn, the parallel use of at least three spawning tanks is strongly recommended. Artificial plants serve as breeding stimulant and substrate. Mating, spawning and fertilization take place within 30 min after the onset of light in the morning.

Since zebrafish is known to feed upon its own offspring, the bottom of the spawning tanks should be covered with a grid of stainless steel (mesh size approx. 2 mm), thus allowing the eggs to be sampled without interference by the adults. The egg trays should be replaced under the spawning tanks at the latest possible time (less recommended) or on the next day before the light is turned on. In the authors´ laboratory, for collection of eggs, the bottom of the 3 L spawning tanks are replaced by a stainless steel grid with a mesh size of 1.25 mm in order to prevent predation of eggs. The spawning tanks are placed on rectangular full-glass dishes of similar dimensions (egg trays; Fig. 1).

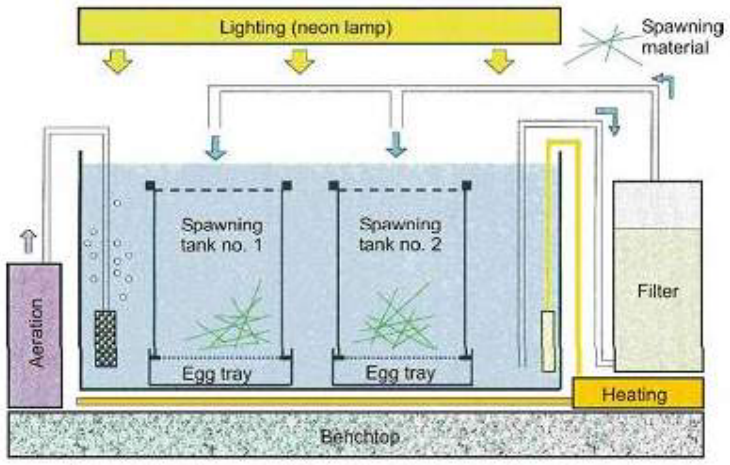

Fig. 1: Tank setup used for breeding zebrafish (*Danio rerio*). Up to 10 tanks, the bottoms of which are replaced by a stainless steel grid, were placed on top of spawning dishes of similar dimensions. All spawning tanks were immersed into one bigger tank equipped with fully conditioned aquarium water. To collect the eggs after spawning, the egg trays can easily be removed from the breeding facility.

As a spawning stimulus, artificial plants made of green plastic or glass should be fixed to the grid covering the egg trays (Fig. 1). About 30 - 60 minutes after spawning, the egg trays can be carefully removed.

For selection of fertilized eggs see 6.3.2.1.